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Title	Examination of the role of the Wilms' tumour suppressor gene (Wt1) in mouse development and neoplasia using a homozygous Denys-Drash syndrome mutation
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Qualification	PhD
Year	2001

Digitisation notes:

- Page number 133 is missing in the original thesis.

Examination of the role of the Wilms' tumour suppressor gene (*Wt1*) in mouse development and neoplasia using a homozygous Denys-Drash syndrome mutation

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Contents

Declaration

The work described in this thesis has been carried out by myself except where otherwise specifically stated.

Kate Wagner
September 2000

Abstract

Denys-Drash syndrome, which is characterised by genitourinary abnormalities, a type of progressive nephropathy known as mesangial sclerosis and the development of Wilms' tumours, results from mutations in the zinc finger-coding region of one copy of the *WT1* gene. Murine ES cells from the CGR8 line with DDS-type mutations in both *Wt1* alleles (compound heterozygotes) were produced by gene targeting and their properties compared with wild-type and DDS heterozygous cells. To distinguish between effects of the *Wt1* mutations and of alterations elsewhere in the genome, cells of the E14 ES cell line and their DDS heterozygous and homozygous derivatives were also studied. While attempting to characterise the mutant cells in terms of RNA and protein expression, it was discovered that undifferentiated ES cells do not express *Wt1*. To enable the detection of mutant and wild-type *Wt1* RNA transcripts and proteins, a previously published method of ES cell differentiation using all trans-retinoic acid was adapted to optimise *Wt1* expression. Examination of *Wt1* expression levels in differentiated cells of the different DDS genotypes revealed a reduction in the level of mutant protein in the DDS heterozygotes and compound heterozygotes compared with that predicted from the relative levels of RNA transcripts.

Embryoid bodies were produced using cells of the above genotypes. Some of these were grown in suspension, others were allowed to attach to the surface of a flask and form outgrowths. A comparison of these embryoid bodies revealed no obvious differences between the differentiation capabilities of wild-type and DDS mutant ES cells. As abnormalites of *Wt1* have been found in leukaemia, including expression of DDS-type mutations, more embryoid bodies were produced, and their potential to differentiate along haematopoietic lineages was assessed using the CFU-A assay. This revealed a delay in the production of haematopoietic cells in *Wt1* mutant embryoid bodies relative to those produced from wild-type cells, which was consistent between the two cell lines examined. Further evidence for this delay was obtained by the examination of the expression patterns of several genes known to be involved in haematopoiesis, using RT-PCR.

To try to identify possible transcriptional targets of the WT1 protein, a comparison of gene expression in retinoic acid-differentiated wild-type and DDS mutant ES cells was performed using expression macro-arrays. Many genes were seen to be expressed at significantly different levels in wild-type and mutant cells, and several of these results were found to be consistent between the CGR8 and E14 cell lines. The differential expression in mutant and wild-type cells of some of these genes was confirmed using RT-PCR analysis. Cyclin G1, heat shock protein 90 alpha and insulin-like growth factor binding protein 4 are as a result suggested as transcriptional targets of WT1.

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List of abbreviations

AGM region	region adjacent to dorsal Aorta, Gonads and Mesonephros
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANLL	acute non-lymphoblastic leukaemia
bp	base pairs
BWS	Beckwith-Wiedemann syndrome
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CFU-A	colony-forming unit A
CFU-C	colony-forming unit culture
CFU-GM	colony-forming unit granulocyte/macrophage
CFU-S	spleen colony-forming unit
CLL	chronic lymphocytic leukaemia
CM/GL	chronic myeloid/granulocytic leukaemia
CR	complete remission
C-terminus	carboxy terminus of protein
DAPI	4,6-diamidino-2-phenylindole
DDS	Denys-Drash syndrome
ddw	double-distilled water
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
e	embryonic day
EB	embryoid body
EC cells	embryonal carcinoma cells
EDTA	ethylenediaminetetraacetic acid
EGF-R	epidermal growth factor receptor
EGR-1	early growth response factor 1
ES cells	embryonic stem cells
EWS	Ewing's sarcoma gene
FACS	fluorescence activated cell sorting
FGF-4	fibroblast growth factor 4
FITC	fluorescein isothiocyanate
FS	Frasier syndrome
FWT1	familial Wilms' tumour gene
G418	geneticin
GAPDH	glyceraldehyde phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
G-CSF-R	granulocyte colony stimulating factor receptor
GM-CSF	granulocyte/macrophage colony stimulating factor
GPI	glucose phosphate isomerase
HPP-CFC	high proliferative potential colony forming cells
HRP	horseradish peroxidase

HSC	haematopoietic stem cell
HSP	heat shock protein
HSV-tk	*Herpes simplex* virus thymidine kinase gene
IFN	interferon
IGF-I	insulin-like growth factor 1
IGF2	insulin-like growth factor 2 gene
IGF-II	insulin-like growth factor 2
IGFBP	insulin-like growth factor binding protein
IL-3,6	interleukin 3,6
INS	insulin gene
IR	insulin receptor
kb	kilobases
kDa	kiloDaltons
KTS region	region consisting of amino acids lysine, threonine and serine
LERK2	Cek 5 receptor protein tyrosine kinase ligand
LIF	leukaemia inhibitory factor
LOH	loss of heterozygosity
M-CSF	macrophage colony-stimulating factor
MIP1α	macrophage inflammatory protein 1 alpha
MMLV	Murine Moloney Leukaemia Virus
MNC	mononuclear cell
MOPS	3-(N-morpholino)propane sulphonic acid
mRNA	messenger ribonucleic acid
neo^r	neomycin phosphotransferase
N-terminus	amino terminus
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF-A	platelet-derived growth factor A gene
PGK	phosphoglycerate kinase
PGPx	plasma glutathione peroxidase
RA	retinoic acid
RAR	retinoic acid receptor
Rb	retinoblastoma gene
RNA	ribonucleic acid
RRM	RNA recognition motif
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SCF	stem cell factor
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	saline sodium citrate
TBE	Tris-borate-EDTA
TCR	T-cell receptor
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor beta

TGS	Tris-glycine-SDS
TNF-α	tumour necrosis factor alpha
TPA	12-ortho-tetradecanoyl phorbol 13-acetate
vol	volume
WAGR syndrome	Wilms' tumour, Aniridia, Genitourinary malformations and mental Retardation
WT	Wilms' tumour
Wt1	murine Wilms' tumour gene
WT1	human Wilms' tumour gene
WT2	second WT locus, at 11p15
YAC	yeast artificial chromosome
ZF	zinc finger

Acknowledgements

I would like to thank a huge number of people for helping me with the work contained in this thesis. In no particular order:

From the MRC Human Genetics Unit, Edinburgh: Aswin Menke for help with immunofluorescence and Northerns; Colin Miles for carrying out the *in vivo* procedures for the teratoma analysis and for letting me steal his PCR primers; and Nick Hastie.

From the John Hughes Bennett Laboratories, Department of Oncology, University of Edinburgh: Helen Taylor for practical help with CFU-A assays; Andrew Cunningham for huge numbers of ideas and explanations of difficult things; and John Ansell.

From the Department of Pathology, Medical School, University of Edinburgh: Jennifer Doig and Tracy Higgins for performing microinjections.

From the Sir Alastair Currie CRC Laboratories, Department of Pathology, University of Edinburgh: Scott Bader for PCR primers and advice; Audrey Peter and Joyce Begbie for technical support; Sarah Plowman, Roberta James and Debbie Bruce for being nice to work with; and Marion Walker for lending me her purple tube rack.

Thanks to the Cancer Research Campaign for providing financial support. Also thanks to all my family, friends and anybody else who has had to put up with me over the last four years. Thanks to Haribo for making the sweeties.

Last, but not least, thanks to Charles Patek and Martin Hooper for all their help and supervision.

1. Introduction

1.1 Gene targeting

Gene targeting is the introduction of planned alterations into the genome by homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences (Thomas and Capecchi, 1987). Techniques developed in recent years have enabled researchers to mutate genes of interest in this way and introduce these mutations into mice. Analysis of the phenotypes of these mice has provided insight into the biological roles of a number of genes.

In 1981, embryonic stem (ES) cells were first derived from the inner cell mass of a mouse blastocyst (Martin, 1981, Evans and Kaufman, 1981). These resemble teratocarcinoma cell lines in that they can be cultured in an undifferentiated state indefinitely. When ES cells are reintroduced to a blastocyst, they can contribute to germline, as well as somatic, tissues (Bradley *et al.*, 1984).

At the same time, it was shown that homologous recombination could be used to target mutations into mammalian cells. In 1987, mutations were first targeted into ES cells (Thomas and Capecchi, 1987), and such mutations were passed through the germline in 1989 (Thompson *et al.*, 1989). ES cells are useful in gene targeting for a number of reasons. Not only are they easy to culture and manipulate by homologous recombination *in vitro*, but they are amenable to a range of selection and screening techniques (Bronson and Smithies, 1994).

The most common strategies for targeted mutagenesis use vectors containing a selectable marker gene, for example neomycin phosphotransferase (*neo*^{r}), which

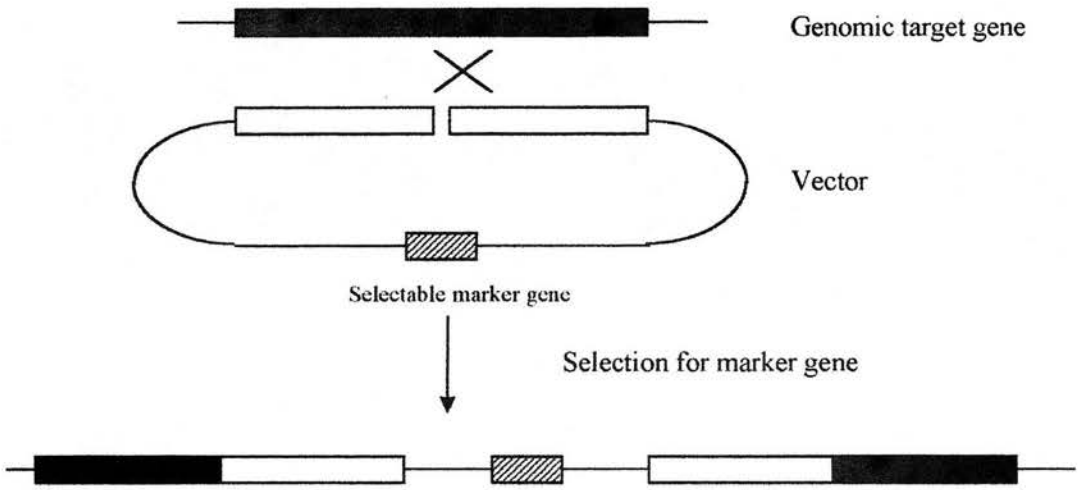
confers resistance to geneticin (G418). This is used to disrupt the gene of interest in a targeting vector *in vitro*. There are two main types of targeting vector, insertion and replacement vectors (Bronson and Smithies, 1994). Insertion (O-type) vectors lead to the duplication of regions of homology contained in the vector. Replacement (Ω-type) vectors insert in place of the DNA segment contained within the regions of homology. These are used for simple gene disruptions (see figure 1.1).

Use of a selectable marker gene enables identification of cells containing the targeting vector, but in the majority of these, the vector will have inserted by non-homologous recombination at random positions in the genome. A positive-negative selection procedure can be used to enrich for targeted events. Mansour *et al.* (1988) described a 2000-fold enrichment for targeted cells, using *neo*^{r} and the Herpes simplex virus thymidine kinase gene (*HSV-tk*), but more recent studies have obtained enrichment in the range 2-20 fold (Hooper, 1992). When the vector replaces the endogenous sequence by homologous recombination, the *HSV-tk* gene will not be transferred. Positive selection for cells containing the vector is by addition of G418. Cells expressing *HSV-tk*, the ones that contain the vector at a non-homologous site, will be killed by the addition of gancyclovir, thus enriching for targeted cells. Presence of the targeted vector is confirmed using PCR and/or Southern analysis.

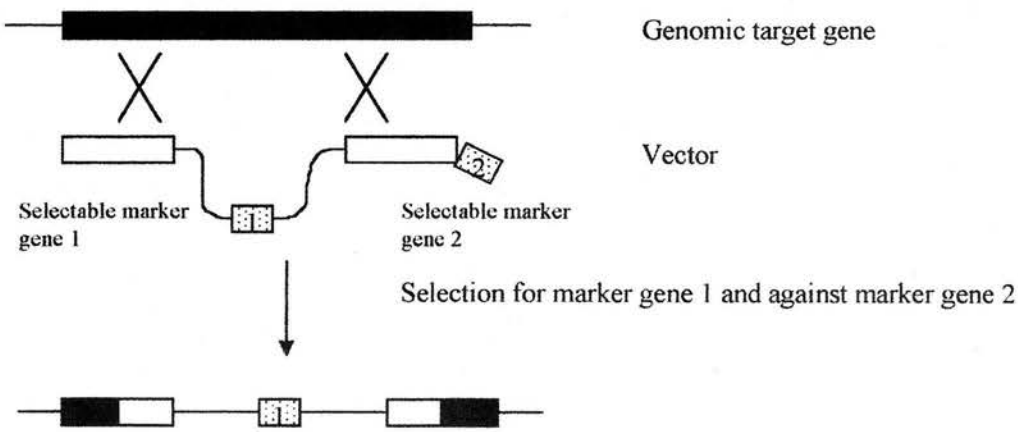
Efficiency of homologous recombination can be increased by increasing the length of the region of homology between the vector and target sequences (Thomas and Capecchi, 1987), and also by using an isogenic vector, derived from the same mouse strain as the ES cells (te Riele *et al.*, 1992). This may increase the targeting efficiency enough to dispense with the use of positive-negative selection.

Figure 1.1 Insertion and replacement targeting events

a. Insertion



b. Replacement

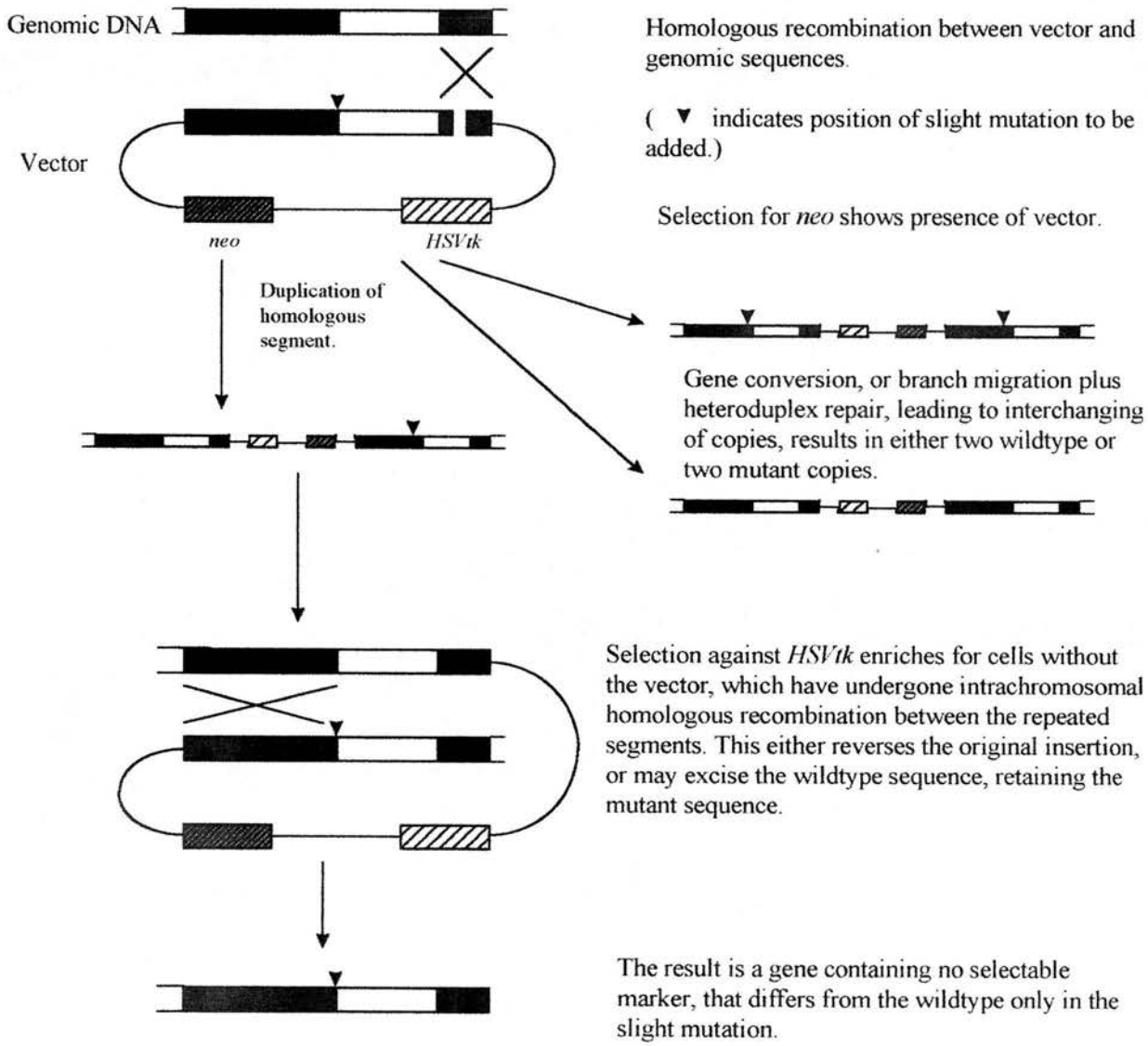


Many germline mutations have been generated which produce a null mutation in the gene of interest. Methods to generate more subtle mutations have been developed, which are useful to increase knowledge of gene function, for example, mutations to determine the function of specific domains within a protein, mutations in sequences controlling gene expression, or mutations mimicking those found in clinical disorders of interest.

The use of selectable marker genes to disrupt the target gene is a limitation, as this gene must be active, and so may affect the mutant phenotype in some way. 'Hit and run' (Hasty *et al.*, 1991) and 'in-out' strategies (Valancius and Smithies, 1991) are similar methods to introduce specific sequence changes to genes without insertion of a selectable marker gene. These involve selection against the marker gene to retain cells out of which the vector has recombined, leaving subtle mutations behind (see figure 1.2). The Cre-lox system uses the bacteriophage P1 enzyme Cre, a member of a family of recombinases (Rajewsky *et al.*, 1996). Cre recognises a 34bp sequence called loxP and will excise a DNA segment flanked by two loxP sites, leaving just one of the loxP sites behind. (Cre will invert a DNA sequence if it is flanked by loxP sites in opposite orientations). A selectable marker gene can be flanked by loxP sites, then removed after selection, by transient transfection of Cre-expression vector into the cell. This system can be used to produce deletions, gene replacements, insertions and point mutations. It also means that once the original marker has been removed, the second allele can be targeted using the same vector.

The Cre-lox system can also be used for conditional (cell-type specific or inducible) gene targeting. This allows the development of models of somatically acquired genetic diseases. If a region of the gene of interest is flanked by loxP sites,

Figure 1.2 Hit and run targeting vector (Hasty *et al*, 1991)(from Hooper, 1992)



this portion of the gene can be deleted in specific cell-types or developmental stages by the induction of Cre recombinase at the appropriate developmental stage or cell type.

Chimaeric mice made by introduction of targeted ES cells into a blastocyst are useful to study the fate of mutant cells in different lineages, and therefore the importance of the gene of interest in development of a particular lineage. The use of chimaeras to study gene function during embryogenesis and postnatal development can have several advantages over constitutional homozygotes or heterozygotes. 'Chimaeric rescue' approaches enable the effects of embryonic lethal mutations to be studied in later development, as rescue by wild-type cells allows development to proceed. In chimaeras consisting of mutant and wild-type cells, competition between the two cell types can be studied, also whether a mutation acts in a cell-autonomous manner, by determining in which cell types the mutation is required in order to produce a given phenotype. Such studies on the *Rb* gene (Maandag *et al.*, 1994, Williams *et al.*, 1994) and the *Pax6* gene (Quinn *et al.*, 1996) have revealed the inability of mutant cells to contribute to tissues in which cell autonomous expression of the particular gene is required during development.

Recessive mutations can often be more effectively studied by creating ES cells which are homozygous for the targeted sequence. There are two main ways of achieving this. Cells can be retargeted using a second vector similar to the first, but containing a different selectable marker, for example a hygromycin resistance gene instead of *neo*^{r}.

Alternatively, if an increased concentration of G418 is added, many of the survivors will be cells that contain two copies of *neo*^{r}, in other words, have become

homozygous for the mutation. This is believed to occur through either chromosome loss followed by duplication, or mitotic recombination (Mortensen *et al.*, 1992). Although high G418 selection is much simpler, as it does not involve the construction of a second targeting vector, a drawback is that the duplication of either the maternal or paternal allele may be accompanied by homozygosity over a substantial chromosome segment, which may in itself produce a phenotype in a gene where imprinting is involved.

1.2 Kidney and Genital System Development

The kidney is the major excretory and osmoregulatory homeostatic organ in the mammalian body. The mammalian kidney develops from the intermediate mesoderm via three successive structures, the pronephros, mesonephros and metanephros (Saxen, 1987).

The first stage of kidney development is the formation of the Wolffian duct, followed by induction of the pronephric tubules from the nephrogenic cord by the Wolffian duct which occurs at embryonic day 22 in humans, E8 in the mouse. The pronephros is transient, and has no function in mammals. The mesonephroi are induced by the Wolffian duct around embryonic day 22 in humans, E9.5 in the mouse.

The ureteric bud forms as an outgrowth of the Wolffian duct, and reciprocal inductive interactions occur between this and the metanephric mesenchyme, during week 5/ E11 (see figure 1.3a). This leads to branching of the ureteric bud and

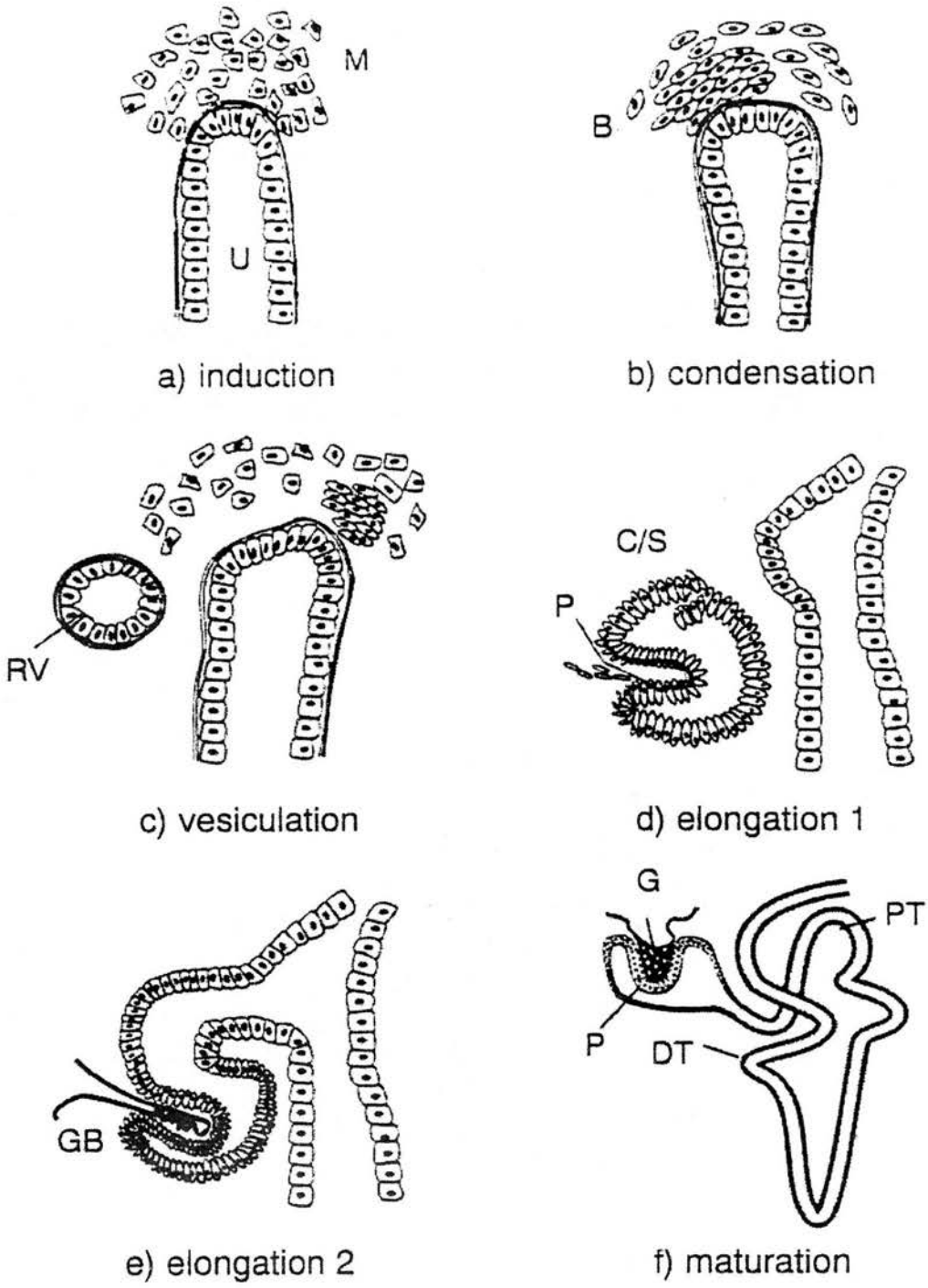


Figure 1.3 Development of the metanephros (from Williamson and Van Heyningen, 1994)

a. Induction U= ureteric bud, M= metanephric mesenchyme
b. Condensation B= metanephric blastema
c. Vesiculation RV= renal vesicle
d. Elongation 1 C/S= comma-/s-shaped body
e. Elongation 2 GB= glomeruloid body
f. Maturation G= glomerulus, PT= proximal tubule, DT= distal tubule

condensation of the mesenchyme into metanephric blastema at the end of each branch (week 5/ E11.5)(figure 1.3b). The metanephric blastema then differentiates into renal vesicles (figure 1.3c), which become comma- and s-shaped bodies (week 7/ E13) (figure 1.3d) then glomeruloid bodies (figure 1.3e) as intermediates in the formation of the mature glomerulus (figure 1.3f). The glomeruli contain mesangial cells and podocytes, which are needed in the mature kidney to maintain glomerular pressure and the flow of filtrate through the kidney.

Gonadal structures develop from the mesonephroi and gonadal ridges. Cells from the degenerating mesonephroi migrate to the gonadal ridge, forming primary sex cords with the germinal epithelium. Primordial germ cells migrate to the gonadal ridge at week 6/E12.

In the male, the Wolffian duct develops into the epididymis, vas deferens and seminal vesicle, with the mesonephric structures incorporated into the testis and other genital tissues. The male genital system is maintained by Leydig and Sertoli cells in the testis. The Mullerian duct in the female forms the fallopian tubes, uterus and upper vagina, with the Wolffian duct and most of the mesonephros degenerating. The remaining mesonephros contributes to the developing ovaries, along with the coelomic epithelium. Granulosa cells in the ovary are responsible for maintenance of the ova. The renal and gonadal systems appear to be far more independent of one another in the female than in the male.

1.3 Haematopoiesis and development of the haematopoietic system

The cells of the haematopoietic system consist of three populations, multipotent haematopoietic stem cells (HSCs), committed progenitor cells, and cells which are mature or maturing.

HSCs are extremely rare, believed to occur at a frequency of 1 per 10^5 nucleated cells in adult murine bone marrow. When haematopoiesis is at a normal, steady state, the vast majority of HSCs are believed to be metabolically inactive, in G_0, with only a few clones responsible for the day to day maintenance of the blood cell population by clonogenic expansion (Lajtha, 1979). The blood system consists of eight cell lineages: platelets, lymphocytes, erythrocytes, macrophages, neutrophils, eosinophils, basophils and mast cells (figure 1.4). These must be replaced by the HSCs throughout an animal's life, as they have finite life-spans. The assay for the ability of HSCs to achieve long-term repopulation of the haematopoietic system is carried out by using the cells to repopulate the haematopoietic system of a lethally irradiated animal (Moore and Metcalf, 1970).

There are two main models for the commitment and self-renewal of HSCs. One sees these as stochastic processes, with very little input from external regulators, such as cytokines, until cells are already committed to a particular lineage or lineages (Ogawa, 1993a). The other is deterministic, with the haematopoietic microenvironment influencing not only the proliferation and survival of committed progenitors, but also the control of lineage commitment itself (Morrison *et al.*, 1997; Metcalf, 1991).

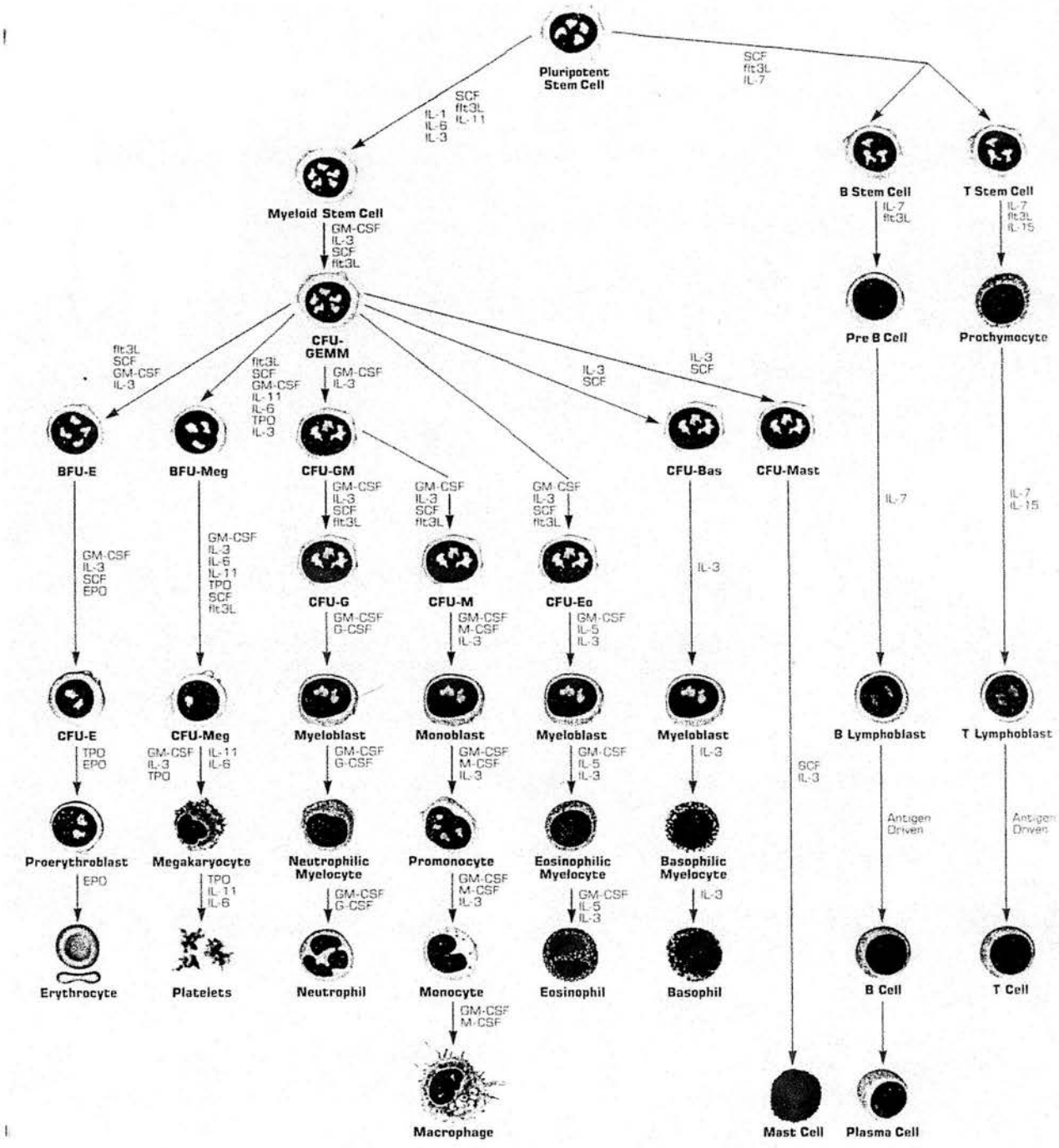


Figure 1.4 The hierarchy of haematopoietic development

Representation of development of the different blood cell lineages from the pluripotent haematopoietic stem cell. Development occurs via intermediate progenitor stages, with cells becoming progressively more differentiated reading down the diagram, until the final, fully differentiated progeny.

Also represented on the diagram are the various cytokines believed to be involved in each stage of the differentiation process.

The immediate progeny of HSCs are called haematopoietic progenitors, or lineage-restricted/committed progenitors. It has been demonstrated that multipotential haematopoietic cells activate several different lineage-specific gene expression programmes simultaneously before lineage commitment actually occurs (Hu *et al.*, 1997). These have more limited potential for further differentiation, and undergo much clonal expansion. The differentiation process from progenitors to mature, terminally-differentiated cells is seen as an irreversible descending hierarchy of differentiation. Cytokines have been demonstrated *in vitro* to be necessary for survival and proliferation of progenitor cells (Han and Caen, 1994).

All haematopoietic cells, including HSCs, are highly influenced by the haematopoietic microenvironment. The bone marrow contains a network of stromal cells, consisting of endothelial cells, macrophages, adipocytes, fibroblasts and extracellular matrix proteins such as collagen, laminin and fibronectin (Lichtman, 1981). The close physical and developmental association between haematopoietic and endothelial lineages within the bone marrow and, earlier, in the yolk sac blood islands, and their common mesodermal origin has led to the suggestion that they arise from a common precursor, which has been named the haemangioblast (Choi *et al.*, 1998).

Non-haematopoietic cells have a role in the regulation of proliferation and maturation of haematopoietic cells via both cell-cell contact and secreted molecules (Clark *et al.*, 1992). Both adhesion proteins and membrane-bound cytokines are involved in binding haematopoietic cells to stromal cells (Ohneda and Bautch, 1997).

The cells of the haematopoietic microenvironment are the major source of predominantly stimulatory (eg. GM-CSF, G-CSF, M-CSF, SCF, IL-3) and inhibitory

(eg. TNF-α, TGF-β, IFN, MIP1α) cytokines. It is a complex equilibrium of these factors within the haematopoietic microenvironment that controls haematopoiesis, including stem cell self-renewal, proliferation, commitment, differentiation and maturation, in a manner that is responsive to changing conditions. Cytokines have been shown to prevent cells from undergoing death by apoptosis (Koury, 1992).

Colony stimulating factors induce clonal proliferation and differentiation. They are characterised by the different types of colonies they induce bone marrow cells to form in assays. Cytokines have pleiotrophic activity, and will work at more than one stage of differentiation, or in more than one lineage. Generally, cytokines acting at later stages of development are more lineage-specific than those acting earlier. For example, erythropoietin is responsible for the maintenance and control of erythropoiesis (Spivak, 1989) and M-CSF only acts in specific macrophage/monocyte lineages (Bajorin *et al.*, 1991). Cytokines such as IL-6, IL-3, SCF and GM-CSF are relatively non-specific (Rapoport *et al.*, 1992; Williams *et al.*, 1990).

Cytokines bind to glycoprotein receptors on the cell surface, activating a signal-transduction pathway which ends with a haematopoietic transcription factor in the cell nucleus. It is suggested that a hierarchical relationship exists among haematopoietic transcription factors (Orkin, 1995), with some, relatively non-specific, acting early in differentiation pathways, eg. SCL/tal-1, Rbtn 2, and others acting later, in a more specific manner, eg. GATA-1, Ikaros, Pu.1. GATA-2 and c-myb are believed to act on HSCs and early haematopoietic progenitors (Tsai *et al.*, 1994; Weston, 1990). In general, most haematopoietic transcription factors seem to act on several different lineages, and it is the combination of transcription factors activated that characterises the lineage (Ness and Engel, 1994). Particular

transcription factors may also play more than one role within a specific lineage, for example, GATA-1 has been found to be responsible for suppressing apoptosis in red cell precursors, as well as regulating the genes that define the erythroid phenotype (Weiss and Orkin, 1995). The first haematopoietic activity is seen at day 7 of murine development. This consists of the differentiation of extraembryonic mesodermal yolk sac cells into primitive erythrocytes (Moore and Metcalf, 1970). At midgestation, haematopoietic activity is found in the foetal liver (E9), thymus (E10.5) and spleen (E15), and does not occur in the bone marrow until just prior to birth (Johnson and Moore, 1975). Movement of the site of haematopoietic activity from the yolk sac to the intraembryonic tissue is not solely due to migration of HSCs from the yolk sac, as haematopoietic cells arise independently in both sets of tissues (Godin *et al.*, 1993; Medvinsky *et al.*, 1993).

Formation of the yolk sac from extraembryonic endodermal and mesodermal derivatives begins around E6.5. Primitive nucleated erythrocytes aggregate in blood islands with endothelial cells from day 7 (Tavassoli, 1991). Evidence for a common precursor to the endothelial and blood cell lineages in blood islands comes from mouse knockouts (Shalaby *et al.*, 1995; 1997).

The primitive haematopoiesis occurring in the yolk sac produces a limited range of blood cell types, confined to erythrocytes and a small proportion of macrophages (Gordon *et al.*, 1992). The erythrocytes present differ from those produced in definitive haematopoiesis in that they are larger, remain nucleated, and express embryonic, rather than adult, haemoglobins.

Formation of the liver begins at late E9, and definitive haematopoiesis is established within it by day 10.5 (Johnson and Jones, 1973). This definitive

haematopoiesis does not actually originate in the liver, but comes from colonising cells via the circulation. The modified model for mammalian haematopoiesis (Dzierzak and Medvinsky, 1995) postulates that colonisation of the foetal liver happens as two separate waves. The first wave comes from the yolk sac at around day 9, and consists of CFU-C (colony-forming unit-culture) cells, that are already committed. The second wave, at day 10, comes from the AGM region (adjacent to the dorsal Aorta, Gonads and Mesonephros) (Godin *et al.* 1993; Medvinsky *et al.*, 1993), and contains primitive CFU-S (see below) and long-term repopulating HSCs. This forms a stable population of definitive HSCs, which replaces the extraembryonically-derived progenitors (Zon, 1995).

Definitive haematopoiesis, which begins in the yolk sac (Palis *et al.*, 1999), then continues in the foetal liver and bone marrow, produces cells of all haematopoietic lineages, with nucleated erythrocytes containing adult globins. The cells responsible for definitive haematopoiesis originate from the dorsal mesoderm of the AGM region, although blood cells from the yolk sac have been shown to be capable of reconstituting the haematopoietic system of lethally-irradiated mice (Moore and Metcalf, 1970; Toles *et al.*, 1989).

The liver never entirely loses its potential for haematopoiesis, and can regain this activity in an emergency, but its haematopoietic activity declines after birth. Colonisation of the thymus by haematopoietic precursors occurs from day 10.5, but this activity again declines around birth (Moore and Owen, 1967). Haematopoiesis in the spleen begins at day 15 and continues until a few weeks post-natal. In mice, the spleen continues to be the site of erythropoiesis. Lymphoid cells are first seen after the development of the thymus and spleen.

The CFU-S assay is used to measure the haematopoietic potential of cells (Till and McCulloch, 1961). The cells are injected into lethally-irradiated recipient mice, and macroscopic colonies on the surface of the spleen are counted 8-15 days later. CFU-S cells themselves, although fairly immature, are not HSCs (Jones *et al.*, 1990). At mouse E10, CFU-S activity is found in both the dorsal aorta and urogenital ridges (Medvinsky *et al.*, 1996).

The bone marrow is the last part of the haematopoietic system to be colonised by haematopoietic precursors, at day 16 (Ogawa *et al.*, 1988). Although responsible for the production of all haematopoietic lineages in adult life, the bone marrow does not gain full haematopoietic potential until after birth, and before that is limited to granulopoiesis (Tavassoli, 1991).

1.4 Wilms' tumour

Wilms' tumour (WT) is also known as nephroblastoma. It is one of the most common solid tumours of childhood, with an incidence of 1 in 10,000, and was first described in 1899 by Max Wilms. 90% of affected individuals are under the age of seven years (Greenwood *et al.*, 1984).

WTs are derived from the metanephric blastema (Machin *et al.*, 1984), and classically display "triphasic" histology, which means that they consist of persistent blastemal cells (undifferentiated), dysplastic tubules (epithelial elements) and supporting stroma. These are found in different proportions and reflect phases of normal kidney development, suggesting that tumour development may be due to an

abnormal differentiation program. WTs may also contain focal areas of mesodermal derivatives, such as smooth/striated muscle, adipose tissue, cartilage or bone (Coppes *et al.*, 1993a).

Most cases of Wilms' are unilateral and sporadic, but bilateral and familial cases also occur. 3% of WT cases present with genitourinary abnormalities (Williamson and van Heyningen 1994), often as part of a recognised clinical syndrome, such as WAGR (Wilms' tumour, Aniridia, Genitourinary malformations and mental Retardation) or Denys-Drash syndrome.

In 1971, Knudson published his two-hit model of tumorigenesis, based on his study of retinoblastoma, another childhood tumour. He observed that the age-dependence of diagnosis of bilateral retinoblastoma was consistent with the hypothesis that a single somatic event was required for tumour onset, while that for unilateral retinoblastoma fitted a model in which two somatic events were required. In 1973, Comings hypothesised that these two events were affecting a single gene in retinoblastoma.

Knudson and Strong (1972) found a similar result from a statistical analysis of WT cases, supporting a two-hit model. The isolation of the *WT1* gene in 1990 and its loss in many WTs suggested that the two hits required are loss or mutation of both alleles of *WT1*. However, as described below, the situation is more complex than in retinoblastoma.

The *WT1* gene was located in 1990 by three different groups. The region 11p13 was associated with WT through deletions found in WAGR syndrome. Call *et al.* (1990) and Bonetta *et al.* (1990) constructed a cosmid and a YAC library respectively and isolated fragments missing in sporadic tumours containing small

deletions. These fragments were both found to contain the same coding region. Gessler *et al.* (1990) found the same locus by chromosome jumping from a CpG island in the region of interest. Soon after, a sporadic unilateral WT was reported containing a mutation in this gene (Haber *et al.*, 1993).

1.4.1 *WT1* mutations in Wilms' tumour

WT1 mutations found in WTs include gross deletions, small insertions or deletions, point mutations, deletions or insertions associated with di- or tri-nucleotide repeats and splicing defects. Some *WT1* mutations lead to a change in WT1 transcriptional function, for example, leading to activation rather than repression from a particular promoter (Park *et al.*, 1993a). Some WTs have been found to overexpress WT1.

WT1 is not imprinted in normal foetal kidney or WTs (Little *et al.*, 1992a). However, in sporadic WTs with loss of heterozygosity at 11p13, only the maternal allele is lost (Pal *et al.*, 1990). Germline and sporadic deletions and point mutations generally arise in the paternal allele (Little *et al.*, 1992b), which may be more susceptible to mutation, perhaps due to a higher mutation frequency during spermatogenesis. Loss of the maternal allele then uncovers the mutation, leading to loss of function.

Nephrogenic rests are non-neoplastic lesions present in 30-40% of kidneys containing sporadic tumours, and nearly all with bilateral tumours (Beckwith *et al.*, 1990). When nephrogenic rests were screened for *WT1* mutations the same somatic mutations were found in the nephrogenic rest as in the tumour in the same kidney. Inactivation of *WT1* may be an early genetic event, leading to the production of

nephrogenic rests, followed by additional genetic hits causing the WT (Park *et al.*, 1993b).

1.4.2 Other loci implicated in WT

WT1 mutations are found in only around 10% of WTs (Hastie, 1994). Tumorigenesis may involve interaction between *WT1* and other, as yet unidentified, loci. One of these, *WT2*, is believed to be associated with Beckwith-Wiedemann Syndrome (BWS), a clinical syndrome involving WT, which is caused by non-random loss of the 11p15 region. Markers found in this region include *H-RAS, IGF2, INS* (insulin), *H19*, a maternally-expressed growth suppressor, which may interact with *IGF2* (Leighton *et al.*, 1995a & b), and $p57^{KIP2}$, a CDK inhibitor, which is expressed from the maternal allele and induces G_1 arrest (Matsuoka *et al.*, 1995).

IGF-2 is a potent mitogen for mesenchymal cells and is involved in kidney development *in vitro* (Yun *et al.*, 1993). It is downregulated in the transition from mesenchyme to epithelium in the kidney and is overexpressed in ~95% of WTs (Ogawa *et al.*, 1993b). It is located at 11p15, making it a possible candidate for *WT2* and is maternally imprinted in most tissues, although this imprinting is relaxed in many WTs (Ogawa *et al.*, 1993b). WT1 regulates expression of *IGF2 in vitro*, via promoter binding (Little & Wells, 1996). *IGF2* overexpression and loss of imprinting have been found in WTs (Ogawa *et al.,* 1993b), as have repression of $p57^{KIP2}$ and *H19* (Thompson *et al.*, 1996; Hatada *et al.*, 1996). Hatada *et al.* found that the reduced expression of $p57^{KIP2}$ in WTs was due to either loss of the maternal allele or aberrant imprinting. BWS patients with uniparental disomy of the paternal 11p15 region have a higher incidence of WT, plus increased levels of IGF-2 and no H19 or

$p57^{KIP2}$ (Henry *et al.*, 1991). It could be the combination of IGF-2 overexpression and lack of H19 and $p57^{KIP2}$ that causes tumours in these patients (Menke and Hastie, 2000). However, as WTs are also found in other overgrowth disorders, e.g. isolated hemihypertrophy, Sotos syndrome (Clericuzio, 1993), Simpson-Golabi-Behemel syndrome (Pilia *et al.*, 1996) and Perlman syndrome (Neri *et al.*, 1984), the overgrowth itself could favour tumour production by increasing the chance of disruption during kidney development (Menke and Hastie, 2000).

WIT-1 is a transcribed DNA sequence which colocalises with *WT1* (2kb upstream) (Huang *et al.*, 1990). It is transcribed in the opposite direction, and is reminiscent of sequences upstream of some proto-oncogenes. *WIT-1* mutations could lead to deregulation of *WT1* expression. So far, it is not known whether *WIT-1* encodes a functional polypeptide. Some *WIT-1* transcripts include exon 1 of *WT1* (Campbell *et al.*, 1994). This suggests that heteroduplex formation between *WT1* and *WIT-1* transcripts may regulate WT1 protein expression. Therefore, mutation or deregulation of *WIT-1* could lead to changes in WT1 expression without *WT1* mutations (Campbell *et al.*, 1994).

Mutation of the *p53* gene may be another mechanism for alteration of WT1 function in the absence of *WT1* mutations, as p53 may be required for WT1 transcriptional repression. (Maheswaran *et al.*, 1993). One study found that all of 34 WTs assayed overexpressed p53 (Lemoine *et al.*, 1992). Mutations in *p53* were also found in 8 of 11 anaplastic tumours. Progression to anaplasia may be associated with loss of p53 function.

LOH for 16q markers is found in almost 20% of WTs (Coppes and Williams, 1994). This may be a secondary event in tumourigenesis, and is often associated with

poor prognosis (Grundy *et al.*, 1994). It is also associated with hepatocellular carcinomas. *WT1* mutations may interact with other WT genes, including ones at 11p15 (*WT2*), and 16q, other tumour suppressor genes or oncogenes, leading to tumour development.

1% of WTs occur in a familial form, as an autosomal dominant trait, showing incomplete penetrance (Matsunaga, 1981). As no WT kindred has yet been found with constitutional mutation of 11p13, 11p15, or 16q, another locus, or loci, are believed to be involved in familial cases, with recent evidence pointing to 17q12-21 as the location of a familial WT gene (*FWT1*) (Rahman *et al.*, 1996). A good candidate gene located in this region is that for Insulin-like Growth Factor Binding Protein 4 (IGFBP-4), which is known to bind IGF-2 (Tonin *et al.*, 1993). However, many WT families show no linkage to 17q12-21. Other regions of possible importance in these families have been identified, including 3q and 20p (Altura *et al.*, 1996) and 19q (McDonald *et al.*, 1998).

1.5 *WT1*

1.5.1 *WT1* gene and protein

The human *WT1* gene spans of 50kb of genomic DNA, and consists of 10 exons, which produce a main mRNA transcript of approximately 3.0kb (Buckler *et al.*, 1991, Call *et al.*, 1990)(figure 1.5). Other WT1 mRNAs of 1.8 and 2.5 kb are also known (Sharma *et al.*, 1992). The murine *Wt1* gene shows 95% homology to human *WT1*.

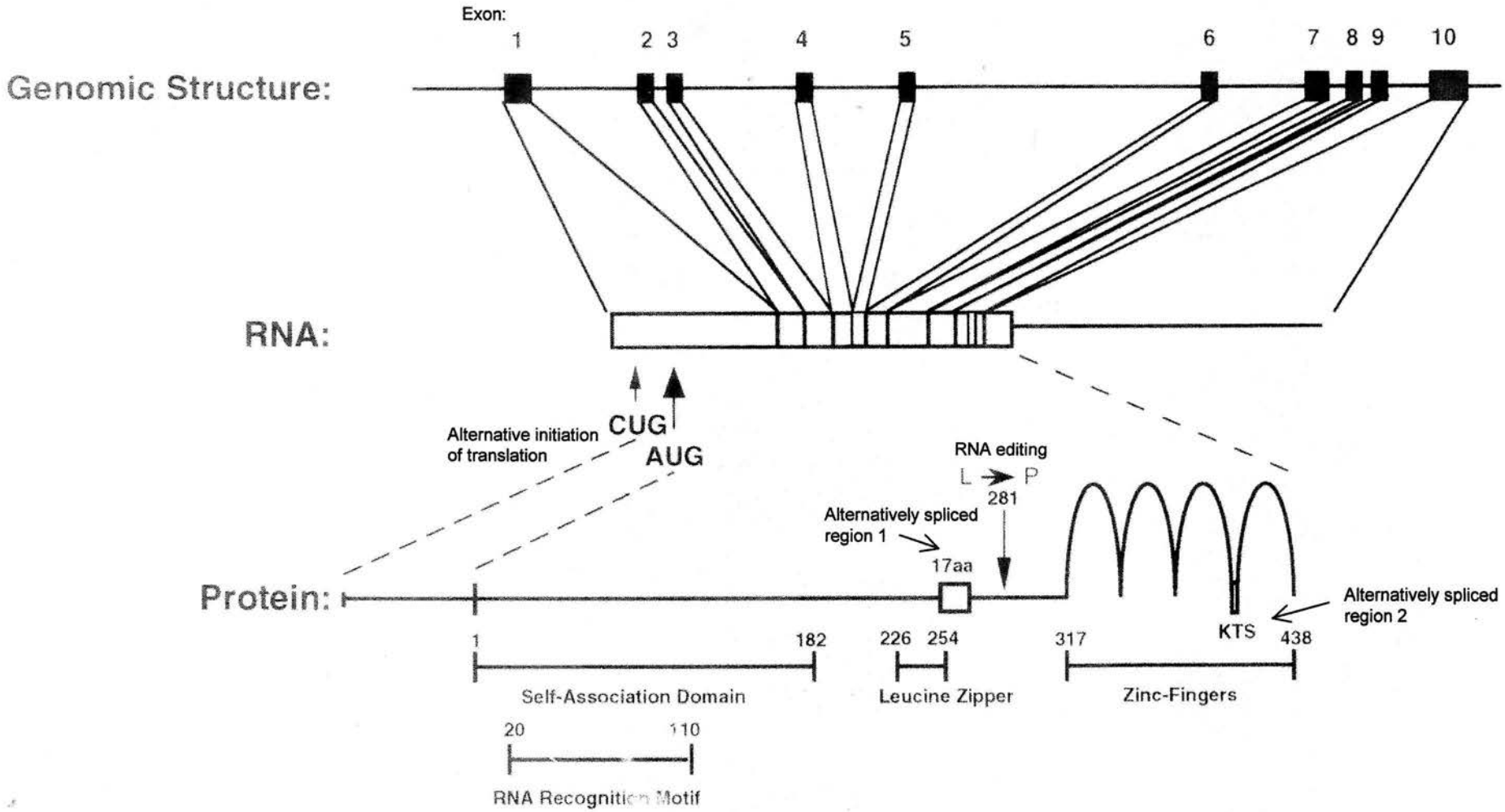


Figure 1.5 The *WT1* gene, RNA and protein

From Menke and Hastie, 2000.

Alternative splicing of *WT1* mRNA transcripts produces 4 different species. These differ in the presence or absence of exon 5 (encoding 17 amino acids), and the presence or absence of a region encoding amino acids lysine, threonine and serine (KTS), in the 3′ region of exon 9.

Further different isoforms of WT1 are produced from an upstream non-AUG alternative translational initiation site, which leads to the production of WT1 proteins of higher molecular weights (Bruening and Pelletier, 1996).

RNA editing occurs in *WT1* transcripts in rats and humans. *WT1* cDNAs have either a T or a C at codon 280, whereas genomic DNA only has a T. The change means that a leucine residue is replaced by a proline (Sharma *et al.*, 1994). This region is not conserved in chickens or alligators, so probably has a mammalian-specific function (Hastie, 1994). Alternative splicing, alternative initiation of translation and RNA editing together produce 16 different isoforms of WT1.

WT1 encodes a nuclear protein, of molecular weight 49-54 kDa, with 4 Kruppel C2-H2 class zinc fingers. It consists of an N terminal proline- and glutamine-rich region and C-terminal zinc fingers. This suggests a role for the WT1 protein as a transcription factor, which is strengthened by the resemblance of 3 of the 4 WT1 zinc fingers to the 3 zinc fingers of transcription factor EGR-1. It is believed that the two proteins may have overlapping sequence specificities, and may be involved in the control of the same genes, possibly in an opposing manner. The N-terminal region and alternatively-spliced exon 5 are not conserved in chicken or alligator, suggesting that either these have a mammalian-specific function, or are not important for WT1 function. The KTS alternative splice is conserved (Hastie, 1994). The signals responsible for localising WT1 to the cell nucleus are believed to be

located in the zinc finger region, but their sequence has not yet been identified fully (Bruening *et al.*, 1996).

Evidence suggests that WT1 expression is controlled at the level of transcription (Grubb *et al.,* 1994). The *WT1* upstream regulatory region consists of a TATA-less promoter, similar to those of *p53* and *RB* (Hofmann *et al.*, 1993). Unlike these, however, *WT1* has highly tissue-specific expression, which suggests that other regulatory elements must be involved. A silencer region has been found in intron three, which does not function in cells of renal origin (Hewitt *et al.*, 1995a). There is a 350bp enhancer 500bp downstream of exon 10 (Fraizer *et al.*, 1994, Wu *et al.*, 1995). This is cell type specific, and contains two GATA motifs, which bind GATA transcription factors. It may also be controlled by a transcriptional silencer, which prevents expression in cell types not normally expressing *WT1.* A 5' cis-regulatory element has been located 15 kb upstream of the *WT1* transcriptional start site, this is conserved between mouse and human, and is necessary, but not sufficient, for transcription of mouse *Wt1* in human cells (Scholz *et al.*, 1997). It remains to be determined which other elements are required for correct temporal and spatial expression of *WT1.*

In vitro experiments have shown that *WT1* transcription is activated by Sp-1 transcription factor (Hofmann *et al.*, 1993). The *WT1* promoter contains binding sites for EGR-1, WT1 and Sp-1 (Fraizer *et al.*, 1994), also NFκB (Dehbi *et al.*, 1998), PAX2 (Dehbi *et al.*, 1996) and PAX8 (Fraizer *et al.*, 1997). There is strong evidence for activation of WT1 by PAX8 *in vivo* in the kidney. Colocalisation, both temporal and spatial, of WT1 and PAX8 is seen, including in WTs. The PAX8 binding site found 250 bp 5' of the minimal human WT1 promoter is not conserved in the mouse,

but PAX8 was shown to activate WT1 expression *in vitro* via this site (Fraizer *et al.*, 1997). WT1 represses transcription of its own promoter in transient co-transfection experiments, so may be controlled by a negative feedback loop. (Campbell *et al.*, 1994).

Evidence also exists for regulation of WT1 by antisense transcripts, which have been detected in 293 cells (Eccles *et al.*, 1994). An 'antisense promoter' was found within intron 1, which is activated by expression of WT1 itself, in a possible feedback mechanism (Malik *et al.*, 1995). The expression of antisense WT1 RNA in 293 cells has been shown to lead to a 50% decrease in WT1 at the protein level.

1.5.2 WT1 protein function

1.5.2.1 WT1 as a transcription factor

The structure of the WT1 protein suggests a role as a DNA-binding transcription factor (Grubb *et al.*, 1994). It is predicted that the WT1 protein should bind to a 12 nucleotide sequence, of the form GNGNGGGNGNGN, where N represents any nucleotide, (Pavletich and Pabo, 1991). Such sequences are found in the promoter regions of many genes that have CpG islands, including some house-keeping genes. As will be seen later, WT1 is expressed at very specific times in very specific locations, and is therefore unlikely to be a regulator of all these genes. Many studies have identified possible transcriptional targets for WT1 *in vitro* (see table 1.1), but as more studies were performed, it became obvious that WT1 transcriptional regulatory activity varies according to the cell system used (Menke, 1998) and type of

expression vector (Reddy *et al.*, 1995a; Wang *et al.*, 1995). Thus, exactly which genes are affected by endogenous WT1 activity remains to be determined.

Table 1.1: Putative *WT1* target genes

Gene	Reference
Insulin-like growth factor 1 receptor (IGF1-R)	Werner *et al.*, 1994
Epidermal growth factor receptor (EGR1-R)	Englert *et al.*, 1995a
Retinoic acid receptor α subunit	Goodyer *et al.*, 1996
Insulin receptor (IR)	Webster *et al.*, 1997
WT1	Hewitt and Saunders, 1996
Early growth response 1 (EGR1)	Wang *et al.*, 1993
Pax2	Ryan *et al.*, 1995
c-myb	McCann *et al.*, 1995
c-myc	Hewitt *et al.*, 1995b
Bcl-2	Hewitt *et al.*, 1995b
MyoD	Miyagawa *et al.*, 1998
Insulin-like growth factor 2 (IGF2)	Ward *et al.*, 1995
Platelet-derived growth factor A (PDGF-A)	Wang *et al.*, 1992
Transforming growth factor β1 (TGF-β1)	Dey *et al.*, 1994
Colony stimulating factor 1 (CSF-1)	Harrington *et al.*, 1993
Inhibin-α	Hsu *et al.*, 1995
Syndecan	Cook *et al.*, 1996
Midkine	Adachi *et al.*, 1996
Ornithine decarboxylase	Moshier *et al.*, 1996
novH	Martinerie *et al.*, 1996
G protein αi-2	Kinane *et al.*, 1996

WT1 has been shown to act as both an activator and repressor of transcription (Madden *et al.*, 1991; Wang *et al.*, 1993). It is believed that WT1 functions as an activator on its own (Reddy and Licht, 1996), but as a repressor when bound to other factors, including p53 (Maheswaran *et al.*, 1993). Which transcriptional function is needed for tumour-suppressor activity has not yet been determined (Reddy and Licht, 1996).

WT1 binds to the same DNA recognition sequence as a related protein, EGR-1, but with less affinity than EGR-1 (30-40 times less). When bound to the EGR-1 consensus sequence, WT1 causes repression of transcription, not activation, suggesting that WT1 has opposite activity to that of EGR-1 (Madden *et al.*, 1991). WT1 has one more zinc finger than EGR-1 does, suggesting that WT1 has its own recognition sequence (Hastie, 1992). Zinc finger 1 (ZF1) may destabilise WT1 binding to the EGR-1 sequence (Rauscher, 1993). WT1 has been shown to repress transcription through a response element in the *IGF2* P4 promoter. EGR-1 activates transcription through the same element (Lee & Kim, 1996). WT1 and EGR-1 have also both been shown to bind to overlapping regions of the *IGF2* promoter 3 (Duarte *et al.*, 1998).

The -KTS form of WT1 can bind to the EGR-1 sequence, but +KTS cannot. The two forms may regulate different genes *in vivo* (Hastie, 1992), with ZF1 playing a role in the different DNA-binding specificities of -/+ KTS isoforms.

-KTS and +KTS DNA binding sequences may have overlapping binding specificities. The -KTS isoforms are believed to bind with high affinity to a 9 nucleotide motif, whereas the +KTS isoforms bind with low affinity to a 12 nucleotide motif (Drummond *et al.*, 1994). The *IGF2* P3 promoter contains a 12bp

sequence that can bind to both isoforms (Drummond *et al.*, 1994). However, in primary embryonic fibroblasts, but not NIH3T3 cells, the + and -KTS isoforms have opposite effects on *IGF2* expression (Duarte *et al.*, 1998). Both isoforms can also bind to the promoters of PDGF-A (Wang *et al.*, 1995), PAX-2 (Ryan *et al.*, 1995) and WT1 itself, causing negative autoregulation (Rupprecht *et al.*, 1994). In +KTS isoforms, all four zinc fingers may be involved in DNA binding, whereas in -KTS isoforms, only zinc fingers 2,3 and 4 are involved (Drummond *et al.*, 1994). All WT1 +KTS binding sites contain a site that can also bind -KTS.

Recent evidence suggests that the presence of the WT1 zinc finger region is not actually necessary for transcriptional repression. A study examining the effect of various forms of WT1 on the IGF1 receptor promoter found that more repression from this promoter was produced by the N-terminal region (including exon 5) alone than the +KTS form of the C-terminus (Tajinda *et al.*, 1999). This may be due to some kind of protein-protein interaction by the WT1 N-terminus. Normal transcriptional repression by WT1 may therefore be due to a combination of DNA-binding by the C-terminal zinc fingers and protein-protein interaction via the N-terminal region.

1.5.2.2 WT1 and splicing

The WT1 protein has been seen to associate with components of the cellular splicing machinery *in vivo* in kidney cell lines, foetal tissue and transfected Cos cells (Charlieu *et al.*, 1995). This is most typical of the +KTS forms, whereas the -KTS forms are mainly found in the transcriptional domains (Larsson *et al.*, 1995). -KTS forms primarily bind DNA, but they can associate with splicing complexes, and will

if DNA targets are unavailable. Does WT1 actually play a role in splicing, or is there just a sequestration of isoforms with lower DNA-binding affinity and non-DNA-binding mutants in sub-nuclear clusters which are separate from those of the cellular splicing machinery? The +KTS isoforms have been shown not to colocalise with SC35, a spliceosome assembly factor (Englert *et al.*, 1995b), but on the other hand, WT1 does interact with U2AF65, a splicing factor (Davies *et al.*, 1998). Disruption of splicing causes the redistribution of WT1 (Larsson *et al.*, 1995), and treatment with RNase changes the subnuclear localisation of WT1, while treatment with DNase does not (Caricasole *et al.*, 1996). A highly conserved RNA recognition motif (RRM) has been located in the N-terminal region of the WT1 protein (Kennedy *et al.*, 1996). Isoforms of WT1 both with and without the KTS insert have been demonstrated to bind RNA from exon 2 of *IGF2*, this may produce downregulation of *IGF2* expression (Caricasole *et al.*, 1996). DNA and RNA binding by WT1 are mutually exclusive, as the binding sites overlap (Bardeesy and Pelletier, 1998). WT1 is not the only Cys2-His2 zinc finger protein to bind to RNA, others, including MOK2, have also been seen to do so (Bardeesy and Pelletier, 1998).

1.5.2.3 Functions of the second alternative splice site

The other alternatively spliced region, exon 5, does not have an effect on DNA binding, but may be important for transcriptional repression functions of WT1 (Madden *et al.*, 1991; 1993). The isoform of WT1 containing both alternatively spliced regions was shown to repress transcription from the *WT1* promoter itself 25 times better than the isoform with the KTS insert, but not exon 5 (Rupprecht *et al.*, 1994). The presence or absence of the 17 amino acid sequence has also been found to

produce proteins having opposite effects. The isoform with both alternatively spliced regions repressed transcription of a PDGF-A promoter construct, whereas the isoform with KTS, but not exon 5, was seen to activate transcription from this construct (Wang *et al.*, 1995). Use of cell proliferation assays revealed that isoforms of WT1 without exon 5 repressed cell growth, isoforms with exon 5 slowed cell growth less, but led to altered cell morphology (Hewitt and Saunders, 1996).

1.5.2.4 Disruption of WT1 isoform ratios and Frasier syndrome

There is evidence to suggest that a disruption of the normal relative proportions of the different WT1 splice variants may itself cause tumorigenicity. One study found an increase in the ratio between transcripts without and with exon 5 when compared with normal kidney in 7 out of 10 WTs (Simms *et al.*, 1995). A similar increase was seen in 4 out of 7 sporadic unilateral tumours (Liu *et al.*, 1999). This could indicate the presence of higher than normal levels of the WT1 isoform lacking both inserts, and, if so, may explain why high expression of *IGF2* has been detected in WTs. This particular WT1 isoform has been shown to induce expression from the endogenous *IGF2* gene in both RM1 (Nichols *et al.*, 1995) and NIH3T3 cells (Hosono *et al.*, 1999).

It has been suggested that an imbalance in the ratios of the + and - KTS WT1 isoforms produces a condition known as Frasier Syndrome (FS). This is characterised by male pseudohermaphroditism and progressive glomerular nephropathy (Frasier *et al.*, 1964). Mutations in intron 9 of *WT1* have been found in FS patients, which prevent formation of + KTS isoforms (Barbaux *et al.*, 1997; Klamt *et al.*, 1998). It was thought that this upset of the normal ratio of the WT1 isoforms somehow

produced the observed pathology. However, two cases of FS have recently been reported with exon 9 mutations which had no effect on the +/- KTS ratio, and another patient was found with no intronic or exonic mutations at all (Kohsaka *et al.*, 1999). This calls into question the role of the imbalanced isoform ratio in the pathology of FS and raises the issue of whether FS is actually a distinct disease from Denys-Drash Syndrome (DDS), which will be described in section 1.6, particularly considering that the exon 9 mutation seen in one of the FS patients had previously been reported in a case of DDS (Ogawa *et al.*, 1993c). However, although the fact remains that this mutation is in exon 9, and is therefore not expected to interfere with splicing of the KTS insert, the patient reported by Ogawa *et al.* (1993) did not exhibit Wilms' tumour, and may in fact have been a misdiagnosed case of FS.

1.5.2.5 Protein-protein interactions of WT1

The WT1 protein is capable of self-association. This occurs via two self-association domains that are located in the N-terminal region of the protein (Holmes *et al.*, 1997).

The WT1 protein has been observed binding to other proteins. It has been found occurring in complexes ranging from 100-669 kDa in size (Maheswaran *et al.*, 1993). The transcriptional activity of WT1 has been found to be affected by physical interaction with p53. WT1 (-KTS) isoforms produce transcriptional repression in NIH3T3 cells, but transcriptional activation in Saos-2 cells, which are p53-null. When wild-type p53 is co-transfected, the observed transcriptional activation is suppressed. However, no difference is seen in the ability of WT1 to bind to the *IGF2* promoter, whatever the p53 status of the cell (Duarte *et al.*, 1998). Other proteins

known to bind WT1 include par-4 (Prostate Apoptosis Response protein 4) (Johnstone *et al.*, 1996), UBC9 (Wang *et al.*, 1996), Ciao 1 (Johnstone *et al.*, 1998) and SF-1. Transient transfection assays revealed that WT1 -KTS isoforms increased transcription of SF-1 more than 20-fold (Nachtigal *et al.*, 1998). par-4 has been shown to increase levels of transcriptional repression controlled by WT1, but reduce levels of transcriptional activation (Johnstone *et al.*, 1996). WT1 also binds to heat shock protein hsp70, via the WT1 N-terminal domain, in embryonic rat kidney cells and cultured cells with inducible WT1, releasing it from complexes with another protein, HSF, which enables HSF-mediated transcription of the hsp70 gene (Maheswaran *et al.*, 1998). Hsp70 has been shown to be induced during haematopoietic differentiation (Sistonen *et al.*, 1992). Interestingly, protein-protein interaction between WT1 and par-4 occurs via the WT1 DNA-binding domain (Johnstone *et al.*, 1996).

1.5.2.6 WT1 in cell proliferation, differentiation and death

WT1 also inhibits DNA replication (Anant *et al.*, 1994), which has been shown to occur through a direct, although as yet unknown, mechanism, as well as indirectly through induction of cell death (Basu *et al.*, 1999). This suggests a direct role in the control of cellular proliferation. It may also play a role in the control of programmed cell death, through a p53-independent mechanism involving suppression of the epidermal growth factor receptor (EGFR) gene (Englert *et al.*, 1995a).

Apparently opposite effects of WT1 on cellular proliferation have been observed when studying different cell lines. If WT (+17 aa) isoforms are injected into synchronised NIH3T3 cells, an inhibition of serum-induced cell cycle progression is

observed (Kudoh *et al.*, 1995). Isoforms both with and without exon 5 have been shown to suppress proliferation in a rat carcinoma cell line (Hewitt and Saunders, 1996), although a stronger suppressive effect was observed with the -17 aa isoforms, with a higher proportion of -17 aa-expressing cells found in G_0/G_1.

WT1 (-17 aa) has been shown to induce expression of p21, a cyclin-dependent kinase inhibitor, in several cell lines (Englert *et al.*, 1997). p21 is expressed in differentiating glomerular podocytes, along with WT1 (Englert *et al.*, 1997). As p21 induces cell-cycle arrest, its induction may be the mechanism by which WT1 prolongs G_1. It has been shown that interaction between WT1 and hsp70 is required for p21 induction in Saos-2 cells (Maheswaran *et al.*, 1998). In a leukaemic cell line, K562, use of WT1 antisense oligonucleotides to suppress endogenous WT1 expression leads to G_2/M arrest (Yamagami *et al.*, 1996; 1998). This means that evidence exists to connect WT1 with two cell-cycle checkpoints, with overexpression of WT1 causing G_1/S arrest, and lack of WT1 leading to G_2/M arrest.

Upregulation of endogenous WT1 expression has been observed when both embryonic stem cells and embryonal carcinoma cells are induced to differentiate using retinoic acid (Scharnhorst *et al.*, 1997). Growth suppression by WT1 requires WT1 to act as an activator, rather than a repressor, of transcription. Three mutant forms of WT1 from WTs, all of which had N-terminal missense mutations, were incapable of suppressing cell growth. These were shown to be capable of transcriptional activation, but not repression (English and Licht, 1999).

In some cell lines, there is an observed downregulation of WT1 when differentiation is induced. For example, HL60 cells will differentiate into

granulocytes when DMSO or retinoic acid is added, into macrophages when TPA is added and into monocytes when vitamin D3 is added. Induction of each of these differentiation programmes is accompanied by a drop in WT1 levels (Sekiya *et al.*, 1994). K652 leukaemia cells can be induced to differentiate into erythroid cells by the addition of sodium butyrate, or megakaryocytic cells by the addition of TPA. Downregulation of WT1 is observed in both these cases (Phelan *et al.*, 1994). Use of antisense WT1 oligonucleotides established that WT1 downregulation is not sufficient for differentiation to occur, but cells stopped growing and underwent apoptosis, suggesting that WT1 expression is important for survival, as well as proliferation (Algar *et al.*, 1996).

In WT1 null mice, the metanephric mesenchyme fails to develop and undergoes apoptosis (Kreidberg *et al.*, 1993). This offers additional evidence for WT1 having a role in cell survival and prevention of apoptosis. However, as in proliferation, the role of WT1 in apoptosis is confusing. As already mentioned, expression of WT1 antisense oligonucleotides in K562 cells led to apoptosis. WT1 is known to suppress p53-mediated apoptosis (Maheswaran *et al.*, 1995), but expression of different WT1 isoforms in Saos-2 and U20S cells was seen to induce apoptosis (Englert *et al.*, 1995a). Only WT1 (-KTS) isoforms caused apoptosis in HepG2 and Hep3B cells (Menke *et al.*, 1997).

The situation is further confused by the fact that many putative target genes of WT1 are involved in apoptosis, both in its inhibition, eg. bcl-2, *IGF2*, and in its promotion, eg. c-myc, c-myb, TGF-β. These opposing effects of WT1 may turn out to be cell-type specific, with WT1 required for survival in some cell types, but involved in switching on genes for apoptosis in others. It is known that the cellular

environment is important in the control of apoptosis, so in some cell types, WT1 may exert an effect on apoptosis by transcriptional control of cell-surface receptors, for example RAR-α, EGF-R, IR.

1.5.2.7 Roles for WT1 in other types of tumour

Abnormal expression of WT1 has been reported in other varieties of tumour (Reddy and Licht, 1996), for example melanoma, leukaemia, mesothelioma, also desmoplastic small round cell tumour, in which reciprocal translocation between the *WT1* and *EWS* genes leads to the production of a fused protein (Karnieli *et al.*, 1996). It is not clear whether *WT1* is involved in other tumours of the urogenital system. WT1 is expressed by 75% of ovarian tumours (Bruening *et al*, 1993), but, to date, only silent, intronic mutations have been found. There may be an effect of *p53* mutation in these tumours (Viel *et al.*, 1994). Polymorphism in WT1 is associated with testicular germ cell tumours. The *WT1* gene is grossly normal in testicular cancers with LOH at 11p13 (Smith and Rukstalis, 1995). No *WT1* mutations have been found in sex-cord stromal tumours (Coppes *et al.*, 1993b). LOH of *WT1* has been found in some cases of bladder carcinoma (Shipman *et al.*, 1993).

1.5.3 The role of *WT1* in kidney development

Studies of *WT1* expression during development provided strong evidence for involvement of this gene in genitourinary development (Pritchard-Jones *et al.*, 1990; Armstrong *et al.*, 1992). *WT1* is expressed at many stages of kidney development, from its onset, right through to the adult kidney (Rackley *et al.*, 1993). *In situ* hybridisation has revealed the presence of *WT1* mRNA in the uninduced metanephric

mesenchyme, then a stronger signal is seen in the induced metanephric blastema and condensations (Armstrong *et al.*, 1992). Presence of WT1 protein is detected in the cell nuclei of the comma- and s-shaped bodies, then later in the podocytes (Grubb *et al.*, 1994).

WT1 expression is also seen in the developing gonad, particularly in the gonadal ridge at the very beginning of gonadal system development. Later, expression is restricted to the Sertoli cells of the testes and granulosa and epithelium of the ovary (Pelletier *et al.*, 1991a).

Other tissues also express *WT1* during development, including the CNS, abdominal wall musculature and mesothelial linings of thoracic and abdominal organs, including the heart (Armstrong *et al.*, 1992). The timing and pattern of expression is consistent with a role for *WT1* in the control of expression of genes involved in mesenchymal differentiation, particularly in the mesenchyme to epithelial transition (Rackley *et al.*, 1993). Expression of WT1 (-/-) in NIH3T3 cells led to induction of epithelial differentiation (Hosono *et al.*, 1999).

During adulthood, *WT1* expression appears to be confined to tumours (Mundlos *et al.*, 1993), supporting cells in the testis and ovary, and the podocytes of the glomerulus. This suggests a role for *WT1* in the maintenance, as well as development of these structures (Reddy and Licht, 1996).

Kreidberg *et al.* (1993) studied *Wt1* function in development by deleting the first exon of *Wt1* plus 0.5kb of upstream sequences. Mice heterozygous for this mutation had no abnormal genital or renal phenotype, and no tumours had appeared by 10 months. When crossed, the resulting live born offspring showed a 2:1 ratio of heterozygotes to wild-types, and no viable homozygotes were found. Homozygous

embryos died between days 13 and 15 of development. On autopsy, these revealed complete failure of kidney development, with no ureteric bud present, indicating a requirement for WT1 in its development, despite WT1 not being expressed in the ureteric epithelial cells (Grubb *et al.*, 1994). The requirement for WT1 must then be in the metanephric mesenchyme. Metanephric blastema was present, but 10-50% of cells showed apoptosis. This subsequently degenerated, and no mesenchymal cells were detectable by day 12. These cells cannot be induced to differentiate by embryonic spinal cord tissue, a strong inducer of mesenchyme into kidney tubules, so the failure of the metanephric blastema to develop is not merely due to the lack of the ureteric duct (Kreidberg *et al.*, 1993). Later, only remnants of the urogenital system were found, consisting of Wolffian ducts in a small ridge along the posterior abdomen. No development of the urogenital ridge occurred after day 11, but migration of the germ cells to the urogenital ridge occurred as normal. The kidney phenotype of WT1-null embryos can be partially rescued by the introduction of the human WT1 gene on a YAC. In some cases nephrogenesis proceeded as far as the s-shaped body stage (Moore *et al.*, 1999a).

During nephrogenesis, WT1 may act on the genes *PAX-2* and *Wnt-4*, expression of both of which is seen in the condensing metanephric mesenchyme just after induction by the ureteric bud. Their expression is down-regulated after the s-shaped body stage of nephrogenesis (Stark *et al.*, 1994; Dressler *et al.*, 1992). PAX-2 null mice develop no kidneys, ureters or genital tracts (Torres *et al.*, 1995). In the Wnt-4 null mouse, no tubular epithelium forms, due to developmental arrest of the metanephric mesenchyme (Stark *et al.*, 1994). It is known that *Wt1* and *Pax-2* can

each regulate the expression of the other, in a possible feedback loop (Ryan *et al.*, 1995; Dehbi *et al.*, 1996).

Death of homozygous *Wt1* null embryos was believed to be due to heart abnormalities, although this was not proven. A generally smaller heart was seen, with a small left ventricle and thin right ventricular walls. The mesothelium was also affected, with incomplete development of the diaphragm, leading to herniation of lung tissue into the abdominal cavity (Kreidberg *et al.*, 1993). Kreidberg *et al.* (1993) suggested that WT1 may be required for the signal from the metanephric blastema to the ureteric epithelium which determines the time and location of ureteric bud outgrowth.

The observation that 5-10% of WTs contain ectopic components, frequently skeletal muscle, led to the suggestion that loss of WT1 expression may lead to the activation of myogenesis (Miyagawa *et al.*, 1998). Five out of seven WTs studied were expressing myogenic genes at high levels, and expression of wild-type WT1 in C2 myoblasts prevented myogenesis on treatment with dexamethasone.

1.6 Denys-Drash syndrome

Denys-Drash syndrome is a congenital nephrotic syndrome, usually leading to end-stage renal failure. Other features are XY pseudohermaphroditism and/or WT. There is evidence for a direct role of *WT1* mutations in DDS, patients being constitutionally heterozygous for *WT1* point mutations. Mutations in the *WT1* gene found in DDS fall into distinct categories, all of which disrupt DNA binding *in vitro.*

Some patients have missense mutations altering amino acids that directly interact with the DNA target. Other affected individuals show substitutions of amino acids involved in zinc complexing, or nonsense mutations leading to the removal of at least two zinc fingers (Little and Wells, 1997). Approximately 60% of DDS patients carry missense mutations in exon 9, the most common of which is a C→T transition, leading to Arg^{394} being replaced by tryptophan in zinc finger 3. This is a mutation hotspot, found in 40% of patients. Such mutations may act by disrupting DNA sequence recognition by the WT1 protein, preventing transcription factor activity. Duarte *et al.* (1998), introduced DDS-type point mutations into both + and -KTS WT1 isoforms and showed that this completely abolished all binding to IGF-2 promoter 3. A patient with the exon 9 mutation inherited it from an apparently unaffected father, suggesting that this mutation exhibits incomplete penetrance (Coppes *et al.*, 1993a). Tumours from DDS patients show loss of heterozygosity (LOH) of *WT1* (Pelletier *et al.*, 1991b).

There is no apparent relationship between the DDS mutation present and the severity of the phenotype seen in the patient. This may be due to plasticity of development in the gonad, or differences in genetic background between patients (Hastie, 1992). Genetic females with DDS are less likely to have genitourinary abnormalities, suggesting that *WT1* may play a more important role in male genitourinary development (Coppes *et al.*, 1993a).

Patek *et al.* (1999) used gene targeting to generate a *WT1* allele with termination of translation within zinc finger 3, producing a truncated protein. In heterozygous ES cells, mutant transcripts are as abundant as the wild-type, but only 5% of WT1 protein found in the nucleus is truncated, suggesting that some form of

post-transcriptional regulation occurs. Mice heterozygous for *Wt1* null mutations develop no obvious kidney pathology (Kreidberg *et al.*, 1993). In contrast, mice heterozygous for the allele truncated in zinc finger 3 exhibit nephropathy characteristic of DDS. (Patek *et al.*, 1999). 90% of DDS patients have WT, but the single *Wt1* DDS heterozygous mouse did not exhibit Wilms'tumour. However, 1 out of 29 DDS chimaeras did. This may reflect the higher risk of WT associated with DDS, compared with WAGR in humans, as the Sey^{Dey} mouse model of WAGR has produced no WTs (Glaser *et al.*, 1990).

WT1 proteins without zinc fingers, and other proteins produced by DDS mutations, can inhibit transcriptional action of wild-type protein. Some of these mutant proteins are completely incapable of DNA binding through the zinc finger region, so most likely do not act in a dominant manner by binding to inappropriate target genes (Little *et al.*, 1993). A dominant-negative mechanism is believed to occur, through protein-protein interaction between mutant and wild-type forms (Reddy *et al.*, 1995b). WT1 has been shown to self-associate, and oligomers between truncated DDS proteins and wild-type proteins form more efficiently or more stably than wild-type oligomers (Moffett *et al.*, 1995). The parts of the WT1 N-terminal domain believed to be involved in self-association have also been shown to be required for the dominant-negative activity of DDS mutant forms (Holmes *et al.*, 1997). Although only 5% of WT1 protein was found to be truncated in the targeted ES cells, the nephropathy observed could be the result of preferential formation of complexes containing mutant and wild-type protein, or to formation of multimers (Patek *et al.*, 1999). DDS proteins bind to RNA with 10-fold less affinity than wild-type WT1 (Bardeesy and Pelletier, 1998).

DDS dominant-negative mutations lead to less than 50% WT1 dosage, but this is enough to allow kidney development (Reddy *et al.*, 1995b). The genitourinary malformations are fairly severe, including streak gonads and pseudohermaphroditism. Patients show progressive nephropathy leading to renal failure, and abnormalities of the podocytes. It has been hypothesised that dominant-negative DDS mutations in the podocytes may inhibit glomerular maintenance and somehow be responsible for the mesangial sclerosis seen in DDS patients. This may occur in association with lack of transcriptional repression of the *PAX-2* gene by mutant WT1. Yang *et al.* (1999) found a distinct lack of nuclear WT1 protein in the podocytes of DDS patients with diffuse mesangial sclerosis and an associated rise in PAX-2 protein, which is normally only expressed up to the S-shaped body stage of kidney development. Mice with dominant gain-of-function *Pax-2* mutations exhibit congenital nephrotic syndrome, with some features resembling the kidney pathology present in DDS (Dressler *et al.*, 1993), so deregulation of the expression of *PAX-2* could be a means by which *WT1* mutations lead to podocyte dysfunction.

It has recently been suggested that at least some DDS mutations may act in a dominant, gain of function, manner, rather than by a dominant-negative mechanism. Tajinda *et al.* (1999) performed transient transfection assays to measure the amount of repression of the IGF-1 receptor promoter produced by various *WT1* mutants. One construct contained the most common, R394W, DDS mutation. Both + and - KTS forms of this protein were observed to repress this promoter in a dosage-dependent manner. Additionally, when the DDS mutant was co-transfected along with wild-type *WT1*, instead of the mutant inhibiting the action of the wild-type, an additive effect on repression was observed, which is the opposite to that which would be expected if

a dominant-negative mechanism were at work. This suggests the possibility that DDS mutants may result in DDS pathology by acting on novel target genes. Alternatively, maybe DDS point mutations have no effect on the ability of WT1 to repress transcription, but only affect its transcriptional activation function.

Another study using PDGF-A and TGF-β reporter constructs in cell lines stably transfected with different *WT1* mutations showed that a different DDS mutation (Cys356Ser) produced less repression than wild-type WT1, but significantly more than mutants lacking some or all of the zinc finger region (Jin *et al.*, 1999).

1.7 WT1 and leukaemia

Leukaemias are defined as neoplastic proliferations of leucocyte precursors in the bone marrow (Anderson, 1985). These are traditionally divided into acute and chronic classes according to the life-expectancy of sufferers, with chronic leukaemia patients surviving for far longer than those with acute leukaemia. Modern methods of therapy mean that this definition no longer applies, but the distinction remains and the two classes can be separated by aetiology, pathogenesis and clinical features. Each class is further divided according to the type of leukaemic cell predominating. Acute leukaemias can be divided into two main groups: acute lymphoblastic (ALL) and acute non-lymphoblastic (ANLL) or acute myeloid (AML). Chronic leukaemias consist of leukaemic cells at more differentiated stages than acute leukaemias, and can be divided into chronic lymphocytic (CLL) and chronic myeloid/granulocytic (CM/GL).

As previously mentioned, *WT1* mutations have been found in several cases of leukaemia. King-Underwood *et al.* (1996) found such mutations in acute leukaemias of both myeloid and biphenotypic types, and discovered that these mutations were associated with a poor response to chemotherapy. A further study by the same group examined 67 cases of leukaemia, finding *WT1* mutations in 14% of AML and 20% of biphenotypic leukaemia patients (King-Underwood and Pritchard-Jones, 1998). However, *WT1* mutations were much rarer in patients with ALL.

Interestingly, seven out of eight *WT1* mutations looked at were found to be in a heterozygous form in leukaemic cells. This suggests either a dominant or dominant-negative mode of action for *WT1* mutations in leukaemogenesis. As well as their heterozygous nature, the mutations found appear to bear a functional resemblance to the mutations found in Denys-Drash syndrome, affecting the zinc finger region, and therefore the DNA-binding ability, of the WT1 protein (King-Underwood and Pritchard-Jones, 1998). DDS patients, however, are constitutional heterozygotes for such mutations, but their tumours almost invariably show loss of the second allele of *WT1*. This increased significance of heterozygous mutations in leukaemic patients suggests that the normal WT1 function in this case is much more easily disrupted by dominant/ dominant-negative mutations than the function which is disrupted to produce WTs. Specific proteins which interact with WT1 in normal haematopoiesis may be prevented from this interaction by the binding of the mutant form to wild-type WT1.

Whatever the function of WT1 in normal haematopoiesis, this evidence suggests that it occurs fairly early on in haematopoietic differentiation, before the splitting of the lymphoid and myeloid lines, as leukaemias of both types contain *WT1*

mutations. WT1 must have some functional redundancy in haematopoiesis, however, as no obvious haematopoietic defects were found in *Wt1* null mice (Kreidberg *et al.*, 1993), and a patient homozygous for a *WT1* missense mutation had WT, but no apparent haematopoietic defects. (Kikuchi *et al.*, 1995).

More studies, however, have found overexpression of normal WT1 in leukaemias. An early study found WT1 expression by Northern analysis in more than 50% of both ALL and AML patients, 80% of CML patients in blast crisis, but no detectable WT1 RNA in other chronic leukaemias, including CML in chronic phase (Miwa *et al.*, 1992). Later, using a more sensitive, RT-PCR, technique, another group found that when patients with leukaemia and with non-Hodgkin's lymphoma were examined, all the leukaemias, including both acute and chronic cases, were found to express high levels of *WT1* mRNA, but no, or very low, *WT1* expression was found in the non-Hodgkin's lymphoma patients (Inoue *et al.*, 1994). It was also found that in CML, WT1 levels increased as clinical phase progressed, and, in acute leukaemias, the level of WT1 expression correlated strongly with the prognosis for the patient, with higher WT1 levels corresponding to a poorer prognosis (Inoue *et al.*, 1994). No mutations in the *WT1* gene were found when these were looked for. Similar overexpression of wild-type WT1 has since been found in children with leukaemia (Niegemann *et al.*, 1999).

Further studies, involving long-term follow-up of leukaemia patients who had achieved complete remission (CR), showed that in patients who later suffered clinical relapse, WT1 levels had either never returned to the levels found in normal individuals, or that WT1 expression had decreased to normal levels then risen again 1-18 months before relapse became apparent (Inoue *et al.*, 1996). Patients who did

not relapse during the course of the study had either no or very low expression of WT1 in their peripheral blood (Inoue *et al.*, 1996). It was later found that WT1 levels in the relapsed patients were, on average, 4.5 times higher than on initial diagnosis (Tamaki *et al.*, 1996).

Inoue *et al.* concluded that WT1 levels in peripheral blood were such a good indicator of possible relapse and effectiveness of treatment that they could be used in patients to monitor minimal residual disease, having the advantage over existing methods of being independent of leukaemia lineage and not relying on the presence of tumour-specific DNA markers.

Efforts have been made to determine which, if any, cells within the haematopoietic system normally express WT1. Bone marrow mononuclear cells (MNCs) were sorted by FACS, according to cell surface antigen expression. Two cell populations were isolated, a more primitive $CD34^+$ population, and a maturer $CD34^-$ population. WT1 expression was examined using RT-PCR, and was found in the more primitive population, but not the more mature (Baird and Simmons, 1997). This corresponds to the fact that more differentiated leukaemias express lower levels of WT1 than less differentiated ones (Inoue *et al.*, 1997). Moreover, when this population was further divided, WT1 expression was seen to be confined to those cells which were $CD34^+/CD38^{-/lo}$, the population believed to contain the haematopoietic stem cell (Baird and Simmons, 1997). This indicates that the role of WT1 occurs very early in haematopoiesis, before much differentiation or lineage commitment has taken place. This would explain why both lymphoid and myeloid tumours are affected by *WT1* mutations. It should be pointed out, however, that several previous studies which had detected WT1 expression in leukaemic cells had

failed to detect any WT1 in normal bone marrow (Brieger *et al.*, 1994), or normal peripheral $CD34^+$ haematopoietic precursors (Mensenn *et al.*, 1995). This could be due in both cases to lower sensitivity of the analysis used.

It is suggested that the downregulation of WT1 is an important step in the differentiation of early haematopoietic precursors, and may even be a prerequisite for this to occur. It has been seen that the induction of neutrophil and monocyte differentiation in the human promyelocytic cell line HL60 and of erythroid/megakaryocytic differentiation of a chronic myelogenous line, K562, are both accompanied by a fall in WT1 expression (Sekiya *et al.*, 1994; Phelan *et al.*, 1994).

MNCs from patients with solid cancers underwent clonal growth assays for various cell lineages. Colonies from these assays were picked after 14, 21 and 28 days, and the WT1 expression was measured by RT-PCR. Expression of WT1 was found only at day 14, not at the later time points, indicating that is a transient phenomenon, occurring only at early stages of haematopoietic differentiation (Mensenn *et al.*, 1997).

If WT1 expression in a certain subset of cells at a specific stage of haematopoiesis is normal, could WT1 expression, rather than being an important part of leukaemogenesis, just reflect normal expression for the cell types present in the leukaemia? This believed not to be the case, as a study found ten times less WT1 expression in normal haematopoietic progenitors than in leukaemic cells (Inoue *et al.*, 1997). The mechanism by which this overexpression occurs is as yet undetermined, but could conceivably function through the action of GATA-1 on the 3' WT1

enhancer, although the expression of GATA-1 is not as widespread in leukaemias as WT1 (Inoue *et al.*, 1997).

Several studies point to the functional significance of WT1 downregulation in the haematopoietic differentiation program and its role in the choice between proliferation and differentiation in early haematopoietic progenitors. Constitutive expression of either + or - KTS isoforms of WT1 in the monoblastic cell line U937 prevented induction of differentiation by retinoic acid or vitamin D3 (Svedberg *et al.*, 1998). This cell line does not normally express WT1. Upon closer examination, some differentiation was seen to have occurred, with the cells arresting in G_1/G_0 upon addition of the inductive reagent, and some changes taking place in cell surface antigens. However, morphological changes did not occur, as seen in untransfected cells, leading to the suggestion that WT1 expression acts as a block to differentiation, not at the beginning of the process, but at a later stage (Svedberg *et al.*, 1998).

A similar result was seen in cell line 32Dcl3, an IL-3 dependent myeloid progenitor line, which will differentiate into neutrophils upon addition of G-CSF. Constitutive expression of WT1 led to cells continuing to proliferate in the presence of G-CSF (Inoue *et al.*, 1998). Again, some differentiation was detected by examination of cell surface antigens, but this was blocked at an early stage (Inoue *et al.*, 1998). WT1 was seen to affect expression of Stat 3α and β, preventing transduction of the G-CSF-R-mediated differentiation signal, but allowing transduction of the proliferation signal (Inoue *et al.*, 1998).

The opposite study was carried out on K562 cells, which do express WT1. Transfection with four out of twenty WT1 antisense oligonucleotides suppressed cell growth in comparison to WT1 sense and random oligonucleotides (Yamagami *et al.*,

1996). These antisense oligonucleotides were also found to have a growth suppressive effect on fresh leukaemic cells from 6/14 AML and 1/2 CML patients, but no effect on the growth of CFU-GM colonies (Yamagami *et al.*, 1996). It was shown that the K562 cells inhibited by WT1 antisense were accumulating in G_2/M, suggesting that WT1 expression is important to enable haematopoietic cells to get past this cell cycle checkpoint (Yamagami *et al.*, 1998).

Baird and Simmons (1997) examined *WT1* expression in bone marrow mononuclear cells. Using RT-PCR analysis on single cells, they found that +/- exon 5 isoforms were being differentially expressed within this population. Some cells expressed both isoforms, others only one. In general, the $CD34^+/CD38^{-/lo}$ population expressed more + exon 5 isoforms, whereas the $CD34^+/CD38^+$ population expressed more – exon 5 isoforms. It was hypothesised that exon 5 isoform usage depends on differentiation status of the cell, as the $CD34^+/ CD38^{-/lo}$ population is believed to be less committed that the $CD34^+/ CD38^+$ population. This finding was called into question, however, by Pritchard-Jones and Renshaw (1997), who suggested that the extreme dilutions of *WT1* RNA used for the PCR may have caused arbitrary amplification of one isoform over the other. Renshaw *et al.* (1997) found an excess of transcripts containing the exon 5 alternatively spliced region in tissues of the haematopoietic system compared with all other human tissues apart from the testis. This excess appears to also be present in the leukaemias and leukaemic cell lines also examined, suggesting that, although the splicing ratios in leukaemic cells differ from those found in other tissues, this is merely a reflection of the pattern of splicing in their cell type of origin, not a novel, tumour-associated phenomenon (Renshaw *et al.*, 1997). This is consistent with findings in the same paper which suggest that the

abnormal splice ratios in Wilms' tumours, as compared with adjacent normal kidney found by Simms *et al.* (1995) are due to the developmentally immature cells from which the tumour originates (Renshaw *et al.*, 1997).

The role played by WT1 in leukaemogenesis is confusing, with *WT1* mutations in some leukaemias suggesting that WT1 is playing its traditional role as a tumour suppressor, whereas the link between WT1 expression and proliferation in some leukaemias and cell lines implying an oncogenic function. It is not known whether WT1 mutation or expression plays a part in leukaemic initiation or progression.

It seems probable that WT1 expression is necessary for proliferation to occur in some cell lines, but the role of WT1 mutations in leukaemia remains to be determined, and may be affecting a completely different function of WT1 altogether.

1.8 Summary

Evidence from the phenotype of *Wt1* null mice (Kreidberg *et al.*, 1993) indicates that the *Wt1* gene product is vital for the development of the genitourinary system. The precise role of this transcription factor in this process, and the target genes through which its function is effected, remain unknown. To help elucidate these functions, this project examines the role of *Wt1* in early developmental processes using embryoid bodies (Martin and Evans, 1975), in which these early stages of development are simulated *in vitro*. Gene targeting was used to produce murine ES cells with mutations of a type found in Denys-Drash syndrome (DDS) in both alleles

of *Wt1*. The resultant mutant proteins are incapable of binding to DNA through the WT1 zinc finger region, and are therefore presumed to have no transcription factor function. As mutations in *Wt1* (King-Underwood *et al.*, 1996), of a similar type to those studied here, and overexpression of WT1 (Miwa *et al.*, 1992) have both been detected in human leukaemias, the CFU-A assay (Pragnell *et al.*, 1988), a technique for examining early haematopoietic development *in vitro*, was utilised to compare *Wt1* mutant and wild-type cells. The mutant ES cells also provided a system for the examination of transcriptional regulation by the WT1 protein without the use of tranfected constructs, with *Wt1* expression under the control of its own promoter. cDNA expression arrays (Kelly and Rizzino, 2000) were utilised to compare expression of 588 different genes in wild-type and *Wt1* mutant cells. As well as allowing the study of many different genes simultaneously, this technique enabled WT1 function to be studied under more physiological conditions than had previously been attempted, producing a more accurate picture of which genes are affected by WT1.

2. Aims and objectives

- To continue the work begun by Patek *et al.* (1999) by generating ES cells with mutations producing DDS-type truncations in both alleles of the *Wt1* gene.

- To use an *in vitro* differentiation approach to examine the role of WT1 in early embryogenesis through production of wild-type and DDS mutant embryoid bodies. This enables development of all three primitive germ layers to be studied at once and assessment of the importance of WT1 in these processes to be made.

- To study in more detail the role of WT1 in haematopoietic development using a specific *in vitro* assay which assesses the potential of embryoid bodies to differentiate along haematopoietic lineages (Hole *et al.*, 1996). This can be used to compare this potential in wild-type and *Wt1* mutant embryoid bodies, and help to define the role of WT1 in haematopoiesis and possibly also leukaemogenesis.

- To identify possible target genes of WT1 using cDNA expression array technology to compare gene expression in wild-type, DDS heterozygous and DDS compound heterozygous ES cells. This has the advantage over previous studies that it employs endogenous expression of both *Wt1* and target genes, and all splice isoforms of WT1 are present. DDS heterozygous cells had

previously been demonstrated to cause mesangial sclerosis in chimaeric mice (Patek *et al.*, 1999), making examination of gene expression in these and the DDS compound heterozygous cells relevant to determining the molecular basis of DDS.

- To use the DDS compound heterozygous cells to produce chimaeric mice, utilising a 'chimaeric rescue' strategy to gain insight into the role of WT1 in mouse development. This exploits the fact that the presence of sufficient wild-type cells in a chimaera allow development to proceed, but information can still be obtained on the effects of the mutation due to the contribution of mutant cells to the chimaera.

- To generate experimental teratomas *in vivo* using wild-type and *Wt1* mutant ES cells. This is an established method for determining the early developmental potential of mutant ES cells (Bloch *et al.*, 1997; Moore *et al.*, 1999b) particularly in cases where the null mutation has been found to be embryonic lethal (Kreidberg *et al.*, 1993). It is assumed that the DDS truncation would be lethal if present in both alleles, as it leads to disruption of the WT1 zinc finger region, but this has not yet been demonstrated.

3. Materials and methods

3.1 Cell culture methods

3.1.1 ES cell lines

The murine embryonic stem cells used were either from the CGR8 (Mountford *et al.*, 1994) or E14 (Hooper *et al.*, 1987) lines. These included wild-type, DDS heterozygous (*Wt1/Wt1*^{tmT396} - CGR8 clones 10 and 15; E14 clones 75 and 261) and DDS homozygous (*Wt1*^{tmT396}/*Wt1*^{tmT396} - E14 clones DK3A and 75/28) (Patek *et al.*, 1999).

3.1.2 Routine maintenance of ES cells

All tissue culture procedures, unless stated otherwise, were carried out in a Class 2 biological safety cabinet. The medium used was standard tissue culture medium (see appendix I) unless otherwise specified.

ES cell stocks were stored in medium containing 10% DMSO above liquid nitrogen. Freezing vials were removed from the liquid nitrogen canister and thawed briefly using water taken from a 37°C water bath. The cells were then centrifuged using a MSE Mistral 1000 hanging bucket centrifuge at 1000rpm for 5 minutes. Unless stated otherwise, this type of centrifuge was used for all ES cell culture procedures. The supernatant was removed by aspiration, then the cells resuspended in fresh medium and transferred into a prepared 25cm^2 flask (Corning Costar) using a plugged pasteur pipette.

Flasks were prepared for ES cell culture by adding 3ml of cold, filter-sterilised 0.1% gelatine (Sigma), and then leaving at 4°C for at least 1 hour. Before use, the gelatine was removed and 7ml of tissue culture medium, prewarmed to 37°C, was added.

After the thawed cells had been placed into the flask, it was left in a 37°C, 5% CO_2 incubator for 2 hours to allow the cells to stick down to the surface of the flask. The medium was then removed by aspiration, to remove dead cells and any

remaining DMSO, and replaced with fresh prewarmed medium. Used medium was removed in this manner at least every second day and replaced with fresh, prewarmed medium.

When the cells became confluent, they were passaged using TVP (see appendix I). The used medium was first removed by aspiration, and the cells washed with approximately 8ml of prewarmed PBS, which was also removed by aspiration. 1ml of TVP was then added to the flask, ensuring that the cells were completely covered. The flask was then placed back into a 37°C incubator for 5 minutes, or as long as was required to detach the cells from the base of the flask. 4ml of tissue culture medium was then added to take up the cell suspension. Approximately 0.5ml of cell suspension was placed into a fresh 25cm^2 flask, prepared as described earlier. The remaining cells were pelleted at 1000rpm for 5 minutes. The supernatant was removed by aspiration, and the cells were resuspended in 3ml of filter-sterilised medium CM (see appendix I), with 10% DMSO. 1ml of suspension was then placed into each of three Nunc CryoTubes (Life Technologies). The vials were put into Nunc cooling racks (Life Technologies) and immediately placed into a -70°C freezer and left there overnight before being placed above liquid nitrogen until required.

3.1.3 Maintenance of M15 cells

Cells from the M15 mouse mesonephric cell line (Larsson *et al.*, 1995) were used as a positive control for *Wt1* expression in RNA and protein applications. These were cultured in the same manner as ES cells, although plasticware did not require gelatinisation, as these cells are able to attach to the plastic itself. The cells were grown in medium CMβ (appendix I), as LIF was not required to inhibit differentiation. Cells were harvested in the same manner as ES cells, and RNA and protein preparation was carried out using the same method (see section 3.1.9).

3.1.4 Preparation of metaphase spreads

A medium sized (75cm^2) flask of cells to be used in the production of metaphase spreads were split approximately 1:20 several days before use. The medium was then

left unchanged until approximately 24 hours before they would reach 60% confluence. The following day, the used medium was removed by aspiration, and 10ml of fresh medium, to which had been added 100µl of KaryoMax® Colcemid® 10µg/ml solution (Life Technologies), was placed into the flask. The flask was then replaced in the incubator for two hours.

After this time, the medium was removed by aspiration, and 2ml of TVP added. After incubation at 37°C for approximately 5min, the flask was shaken gently to create a single cell suspension. 8ml of medium was then added, and the total 10ml was centrifuged at 1000rpm for 5 min, to pellet the cells. The supernatant was removed, then the cells resuspended in 10ml 75mM KCl, and left at room temperature for 15min. At this point, the cells were again centrifuged at 1000rpm for 10 min. The KCl was removed by aspiration, and approximately 1ml ice cold fixative (appendix I) added, drop by drop, using a pasteur pipette with shaking in between each drop to prevent clumping of the cells. A further 9ml of ice cold fixative was then added slowly, after which the cells were left to fix at room temperature for 10 min, before being pelleted by centrifugation at 1000rpm for 5 min. The fixative was removed by aspiration, and the cell pellet resuspended in a further 10ml of ice cold fixative. The cells were then pelleted again, as above, but this time 1ml of fixative was left behind after aspiration. The cells were gently resuspended, then dropped onto cold microscope slides, which had been stored at 4°C in 100% ethanol. The slides were left to dry for a few minutes, then labelled with a diamond pen and stained for 15 min with 5% Giemsa in PBS. After staining, the slides were rinsed in water to remove excess stain. They were then left until dry, when cover slips were attached using DPX (Merck).

3.1.5 Electroporation of ES cells

Cells to be transformed by electroshock were grown to confluence in a 162cm^2 flask, as described in section 3.1.1. These were then washed in PBS and detached from the flask using 3ml of TVP. The resulting single cell suspension was taken up in a further 7ml of warmed tissue culture medium, after which the cell concentration was measured using an Improved Neubauer haemacytometer (Fisher).

A volume calculated to contain approximately 1 x 10^8 cells was centrifuged in a hanging bucket centrifuge at 1000rpm for 5min, then the supernatant removed by aspiration.

In the meantime, the DNA with which the cells were to be transformed was prepared. 150µl of the WTV2 plasmid had been linearised by digestion with restriction enzyme *Not*I (see section 3.2.4), then precipitated for 1 hour at -70°C with ½ volume of 7.5M ammonium acetate and 2 volumes of absolute ethanol. The DNA pellet was obtained by centrifugation in a microfuge at 13,000rpm for 10 min. After a wash with 80% ethanol, the pellet was air dried, then resuspended in 0.6ml of sterile PBS in the tissue culture safety cabinet.

The DNA suspension was used to resuspend the ES cell pellet. The cell and DNA suspension was then transferred into a 1ml cuvette (BioRad, path length 0.4cm), which was inserted into the Bio-Rad gene pulser, and an electric pulse of 800mV with a capacitance of 3µF was applied. The cuvette was then left at room temperature for 10min before being added to 100ml of tissue culture medium which was then divided between ten 100mm tissue culture dishes. The cells were placed into a 5% CO_2 incubator at 37°C overnight before the selection procedure was started.

3.1.6 Selection of targeted ES cells

The day following electroporation with the WTV2 plasmid, the medium was replaced with medium containing 100µg/ml G418 (Life Technologies) and 100µg/ml hygromycin B (Sigma) which had been prewarmed to 37°C. After a further two days of incubation, the selective medium was replaced with fresh selective medium. After a further two days, the medium was removed again and replaced with selective medium with the addition of 2 x 10^{-6}M ganciclovir (Roche)(Mansour *et al.*, 1988). This medium was then replaced after three days, then, after a further two days, it was replaced with medium containing just G418 and hygromycin.

When ES cell clones growing on the plates were seen to be large enough, they were picked under a light microscope in a still air hood, then each placed into a drop of TVP in a petri dish for a few minutes to dissociate the cells. The drops of TVP

were each then added to 3ml of selection medium in one well of a gelatinised 24 well plate (Corning Costar). The selection medium in the wells was changed every two days until the well became confluent. At this point, the cells were trypsinised and transferred into 2 wells of a gelatinised 24 well plate in selective medium, which was changed every second day until the wells became confluent. At this point, the cells were again removed from the well using TVP. One third of the cells were frozen down, as described in section 3.1.2. The remaining two thirds of the cells were transferred in medium into a 1.5ml microfuge tube, centrifuged in a microfuge at 4000rpm for 5min, then stored at -20°C after the supernatant was removed using a pasteur pipette. DNA extraction was then carried out as described in section 3.3.1.

3.1.7 Preparation of ES cell pellets for DNA extraction

A confluent 25cm^2 flask of ES cells was trypsinised as described in section 3.1.2 to obtain a single cell suspension. This was taken up with 4ml of tissue culture medium and then centrifuged for 5 minutes at 1000rpm. The supernatant was removed, then the cells were resuspended in 1.5ml of tissue culture medium and transferred into a 1.5ml microcentrifuge tube. This was then microfuged at 4000rpm for 5 minutes to pellet the cells. The supernatant was removed, and the pellet stored at -20°C until required for DNA preparation, as described in section 3.3.1.

3.1.8 *In vitro* differentiation with retinoic acid

Cells to be differentiated with retinoic acid were grown to confluence in 162cm^2 flasks, then split 1:50 into 162cm^2 flasks. These were then grown in normal tissue culture medium for 2 days, after which the medium was replaced with medium containing 5μM all-trans retinoic acid (Sigma) and β-mercaptoethanol, but no LIF. The retinoic acid was dissolved in DMSO, which had a final concentration in the medium of 0.05%. The medium was removed and replaced with freshly made up medium every day for four days. After this time, the cells were harvested for either RNA or protein preparation, as described in section 3.1.9.

3.1.9 Preparation of cell pellets for RNA and protein extraction

Cell harvesting for RNA extraction was carried out using disposable cell scrapers (Greiner). The medium was removed from the cells, and the cell scraper used to detach all the cells from the base of the flask. Approximately 10ml (for a 162cm^2 flask) of tissue culture medium was then used to take up the cells, which were then centrifuged at 1000rpm for 5 minutes to produce a pellet. The supernatant was removed by aspiration and 5ml of ice-cold PBS used to wash the cell pellet, which was then centrifuged again at 1000rpm for 5 minutes.

Cells for immediate use were then resuspended in a further 1.5ml of ice-cold PBS and transferred into a 1.5ml microfuge tube. This was then centrifuged at 5000rpm at 4°C in a Heraeus Biofuge Fresco (Fisher) for 5min, after which the supernatant was removed by aspiration and the extraction procedure begun.

Pellets for RNA extraction which were to be stored for a time were first resuspended in 1ml of ice-cold PBS and transferred into a freezing vial. This was centrifuged at 1000rpm for 5min, then the medium was removed and the pellet immediately snap-frozen in liquid nitrogen. Pellets were stored over liquid nitrogen until RNA extraction was performed as described in section 3.4.1.

Pellets to be stored for protein extraction were transferred into 1.5ml tubes, as for fresh pellets, then the supernatant was removed by aspiration and the pellets stored at -70°C until required. Protein extraction was then carried out as described in section 3.5.1.

3.1.10 *In vitro* differentiation into embryoid bodies - cell aggregation method

Single cell suspensions of ES cells were seeded into 60mm tissue culture dishes (Corning Costar) at 3 x 10^6 cells per dish in medium EFNβ. The medium was changed every day for approximately 4 days, until the cells were very confluent. At this point, the used medium was removed using a sterile wide bore pipette tip and transferred into a sterile 20ml universal tube. 5ml of fresh EFNβ was used to dislodge clumps of cells from the surface of the dish by squirting through a narrow-bore pipette. The cell clumps were taken up gently using a wide bore pipette tip and

transferred into the same universal tube as the spent medium. This was repeated using two more 5ml volumes of fresh medium. The universal tube was left undisturbed for approximately 5min, until the cell clumps were seen to sediment under gravity, then all but approximately 1ml of the medium was carefully removed by aspiration. A wide bore pipette tip was used to resuspend the clumps in 10ml of fresh medium EFNβ.

The cell clumps were then placed into dishes that had been prepared on the previous day, one 100mm dish (Corning Costar) per previous 60mm dish. Solutions of 1% and 2% low melting point agarose (Sigma) were made up in PBS and melted by autoclaving. These were kept at 65°C in a water bath until required. 100mm tissue culture dishes were coated with 2% agarose by adding 10ml of melted agarose and swirling to cover the entire surface, then removing 7ml. The agarose remaining in the dish was left to set at room temperature. A layer of 1% agarose was then added on top of the first layer in the same manner. After the second layer of agarose had set, 10ml of EFNβ was placed into the dish, which was incubated at 37°C overnight to equilibrate. The equilibration medium was then removed and replaced with the medium containing the cell clumps. The dishes were then incubated at 37°C in a tissue culture incubator, with medium changes every two days. This was achieved by removing the used medium containing the cell aggregates with a wide-bore Gilson tip into a sterile 20ml universal tube, and allowing the aggregates to precipitate under gravity. The supernatant was carefully removed and replaced with fresh medium, and the cell aggregates were then returned to the agarose-coated dish.

Cell aggregates were either left to differentiate in suspension in agarose-coated dishes for up to 28 days, or were transferred into non-coated dishes to attach, after approximately four days in suspension culture. Aggregates which remained in suspension were harvested by removing from the dishes into universal tubes, allowing to sediment, removing the supernatant, washing twice in PBS and fixing in Bouin's fluid (see appendix I). Cell aggregates for attachment were transferred into universal tubes as described above, then resuspended in 16 ml of medium CMβ. This was divided between four 60mm non-gelatinised tissue culture dishes. The dishes were placed into a 37°C incubator and left undisturbed for 48 hours to allow the

aggregates to attach to the dishes. The medium was changed by aspiration and replaced with fresh CMβ every 2 days.

3.1.11 *In vitro* differentiation into embryoid bodies - hanging drop method

Medium used for the production and differentiation of hanging drop embryoid bodies was the same as standard tissue culture medium (appendix I), except that 10% foetal calf serum was used instead of 5% foetal/ 5% newborn calf serum.

Approximately 10ml of sterile water was placed into each of a quantity of 90mm square petri dishes (Life Technologies) (approximately 6 dishes per 10ml of drops to be produced). The cells, which had been grown in a $25cm^2$ flask, were trypsinised with 1ml of TVP to obtain a single-cell suspension, then 4ml of medium was added, and the cells were pelleted by centrifugation at 1000rpm for 5min before being resuspended in 5ml of fresh medium. The cell concentration was determined using an Improved Neubauer haemacytometer (Fisher), and 2×10^6 cells added to a gelatinised $25cm^2$ flask, with fresh medium. 3×10^5 cells per 10ml of drops to be made were put into a fresh tube, and the volume made up to the required amount with medium including LIF and mixed well to produce an even suspension of cells. Cell suspensions were placed into a 37°C incubator until required.

10µl drops were pipetted onto the underside of the lids of the prepared square petri dishes using an electronic multipipettor (Wolf Laboratories) until all the cell suspension was used. Petri dish lids were replaced, and the dishes placed in a 5% CO_2 incubator at 37°C for 2 days before harvesting by removing the lid and tapping one corner on the floor of the tissue culture hood. Drops were collected from the corner of the lid using a sterile plugged pasteur pipette and transferred into a 20ml universal tube, then centrifuged at 800rpm for 3 minutes. The supernatant was removed, and the embryoid bodies resuspended in 10ml of differentiation medium per original 10ml of hanging drops. Penicillin/ streptomycin (Life Technologies – penicillin 10,000U, streptomycin 10,000µg/ml) was added at a dilution of 1:100, but no LIF was added to the medium. The embryoid bodies were then transferred into 60mm bacteriological grade petri dishes, with a maximum of 10ml of embryoid body suspension going into each dish. Every 2 days, the medium in the dishes was changed

by removing the used medium and embryoid bodies into a 20ml universal tube, leaving them to sediment by gravity for a few minutes, then the old medium was removed and the embryoid bodies resuspended in fresh differentiation medium, without LIF. The embryoid body suspension was then placed into a fresh 60mm bacteriological grade petri dish for the next 2 days.

When harvesting embryoid bodies for analysis, they were removed from the petri dish into a 20ml universal tube in the manner described above. Embryoid bodies to be used in RNA extraction were washed in cold PBS before being transferred into an RNase-free 1.5ml microfuge tube, then treated in the same manner as ES cells (see section 3.4.1).

3.1.12 Staining of embryoid bodies for the presence of haem

Embryoid bodies produced by hanging drop culture were stained for the presence of haem using *o*-dianisidine using a method adapted from Huber *et al.*, 1998. Embryoid bodies were placed into 0.14% *o*-dianisidine (Sigma) in a 20:25:1 (by volume) mixture of 100% ethanol, 0.2M sodium acetate and 30% hydrogen peroxide in a 20ml universal tube and left for approximately 15 minutes until a red colouring was seen to appear. The embryoid bodies were then washed twice in PBS before being fixed in 4% paraformaldehyde in PBS.

Scoring was carried out by phase contrast microscopy, with the proportion of embryoid bodies with any red staining counted for 5 fields of view.

3.1.13 CFU-A assay

Embryoid bodies were prepared using the hanging drop method, as described in section 3.1.10. After differentiation in suspension culture in the absence of LIF for the required length of time, embryoid bodies were harvested into universal tubes in the manner described above. The supernatant was removed, leaving the embryoid bodies in approximately 1ml of medium. A 10µl aliquot of the embryoid body suspension was placed on a glass slide, and the number of embryoid bodies present counted under a microscope. CFU-A assays were prepared in triplicate, and 50

embryoid bodies were placed into each dish, so a volume of embryoid body suspension containing approximately 150 embryoid bodies was transferred into a fresh tube and were returned to an incubator until required.

Dishes were prepared as described in Pragnell *et al.*, 1988. A layer of agar containing growth supplements was poured into 35mm suspension culture dishes (Corning). To 20ml of 2x stock (see appendix I) was added 20ml of molten 1.2% Noble agar (Difco)(kept at 55°C in a water bath), 4ml (1/10 volume) L929 conditioned medium and 2ml (1/20 volume) AF1 conditioned medium (preparation described in Pragnell *et al.*, 1988). These were quickly mixed, then 1ml was placed into the bottom of each of the required number of 35mm dishes, which were then left at room temperature until the agar had set. In the meantime, the top layer of agar was prepared. This contained 20ml of 2x stock, 10ml of 1.2% agar at 55°C and 10ml of sterile distilled water. After mixing, 3ml was used to resuspend the embryoid bodies, then 1ml of agar and embryoid bodies was placed into each of 3 prepared dishes. The dishes were left at room temperature until the top layer of agar had set, then placed into a 37°C incubator with 5% O_2 and 10% CO_2 for 8-10 days.

CFU-A assay dishes were scored using phase contrast microscopy, and an acetate 1mm^2 grid used to estimate of the size of the colonies. Colonies were scored as being CFU-A positive if they showed the characteristic 'halo' appearance (see figure 5.4) and had a diameter greater than or equal to 1mm. All other colonies and embryoid bodies were scored as negatives.

3.1.14 Secondary replating of CFU-A colonies

Replating of the colonies produced in the CFU-A assay was performed after the colonies had been growing in the assay for 8 days. Whole colonies were picked out, dissociated, and each plated out in a separate new dish. The bottom layer of agar was prepared as described for the original CFU-A assay. While this was setting, CFU-A positive colonies were located under a light microscope and picked out of the agar. The pipette tip containing the colony was placed into a sterile universal tube.

The top agar was then prepared as described in section 3.1.13, and 1ml used to produce a single cell suspension of the CFU-A colony. This agar was then placed

into a prepared 35mm dish, containing a bottom layer of agar. When the top layer had set, the dish was placed in a 37°C incubator with 5% O_2 and 10% CO_2 and left for 10 days.

3.2 Vector and probe preparation

3.2.1 Plasmid mini-prep

Plasmids used during the course of this project were:

1. **WTV2** – constructed by Sheila Christie and Anthony Brookes (see figure 4.3). Used for targeting of DDS heterozygous ES cells and also for preparation of the WTV2 hygro probe, used in Southern analysis. This was prepared by restriction digestion of WTV2 with enzymes *Eco*RI and *Hind*III, followed by recovery of a 1.2kb fragment, as described in section 3.2.6.
2. **pUHD172-1neo** (Gossen *et al.*, 1995) – a 2.7kb fragment (neo) was prepared from this plasmid by digestion with *Xho*I and used for Southern analysis.
3. **pJ5ΩWT1** (Miyagawa *et al.*, 1998) – contains *Wt1* cDNA possessing both alternatively spliced regions. Used as the probe for *Wt1* for Northern analysis. A 1.8kb fragment was recovered after digestion with *Hind*III and *Xba*I.
4. **pBluescript® SKM13(-)** (Stratagene; Leader *et al.*, 1986) – contains mouse skeletal muscle actin cDNA. Used to make probe for Northern analysis. Digestion with *Xho*I and *Eco*RI excises 1.5kb insert.

Preparation of small quantities of plasmid was carried out using a Wizard™ *Plus* Minipreps DNA purification System (Promega), according to the manufacturer's instructions. Bacteria containing the plasmid of interest were inoculated into 10ml of L-Amp broth (see Appendix I) and grown overnight in a 37°C Gallenkamp orbital shaker at 220rpm. 3ml of this culture was then centrifuged at 10,000 x g for 2 minutes in a Sorvall RT 6000D centrifuge (Fisher) to pellet the cells. The cells were then completely resuspended in 200µl of Cell Resuspension Solution and transferred into a 1.5ml microfuge tube. 200µl of Cell Lysis Solution was then added, followed

by 200µl of Neutralising Solution. The lysate was then microfuged in a MSE MicroCentaur at 10,000 x g for 5 minutes.

Plasmid purification was performed using a Vac-Man® Laboratory vacuum manifold (Promega) and the minicolumns provided with the DNA purification kit. The cell lysate was placed into the column, along with DNA Purification Resin, then washed with Column Wash Solution. The syringe barrel was then removed, and the minicolumn inserted into a 1.5ml microfuge tube, into which the DNA was eluted with TE buffer by microfugation. The eluted DNA was then stored at -20°C until required.

3.2.2 Transformation of competent bacteria

The cells used for transformation were Library Efficiency DH5α™ Competent Cells (Life Technologies). 20µl of cells was aliquoted into a chilled microfuge tube. 1µl (1-10ng) of DNA was added and gently mixed. The tube was then placed on ice for 30 minutes before being heat-shocked for 40 seconds in a 42°C water bath, then replaced on ice. 80µl of S.O.C. medium (see appendix I) was added, then the tube was placed into an orbital shaker and shaken at 225 rpm at 37°C for 1 hour. All the cells were applied to one LB-Amp plate (appendix I), then incubated overnight at 37°C.

3.2.3 Qiagen plasmid maxi-prep

Large scale production of plasmid was performed using a Qiagen Maxi plasmid kit. 100µl of bacteria glycerol stock was inoculated into 8ml of L-Amp broth. This was placed in an orbital shaker at 220rpm at 37°C overnight. This was then added into 500ml of L-Amp broth, which was incubated at 37°C at 180rpm in an orbital shaker overnight.

The bacterial culture was transferred into two 250ml centrifuge beakers then centrifuged at 3200rpm in a Sorvall centrifuge for 30 minutes. The supernatant was tipped away, and the bacterial pellet resuspended in 10ml of buffer P1. The bacterial

suspension was then transferred into a 30ml Sorvall centrifuge tube, 10ml of buffer P2 were then added and mixed in, before being incubated at room temperature for 5 minutes. 10ml of chilled buffer P3 was then added, and mixed in immediately. The bacterial suspension was then placed on ice for 20 minutes. After this, the suspension was mixed, then centrifuged at 4,000rpm in a Sorvall centrifuge for 30 minutes at 4°C. In the meantime, a Qiagen-tip 500 column was equilibrated by applying 10ml of buffer QBT, and allowing the column to empty by gravity flow. The supernatant from the bacterial suspension was removed immediately after centrifugation, and applied, through a metal tea strainer, to the Qiagen column. After the supernatant had entered the resin of the column, the column was washed twice with 30ml of buffer QC. The DNA was then eluted from the column into a fresh Sorvall centrifuge tube, using 15ml of buffer QF.

0.7 volumes of room temperature isopropanol was mixed with the eluted DNA, which was immediately centrifuged at 6,000rpm for 1 hour in a Sorvall centrifuge at 4°C. After removal of the supernatant, the DNA pellet was then taken up in 1ml of 70% ethanol and transferred into a microfuge tube. The DNA was then microfuged at 13,000rpm for 5 minutes, after which time the ethanol was removed and the pellet left to dry at room temperature for approximately 15 minutes before being resuspended in 300µl of TE buffer (appendix I) and stored at -20°C.

3.2.4 Restriction enzyme digestion of plasmid DNA

Restriction enzyme digests were performed according to the manufacturer's instructions, using the restriction buffer supplied with the enzyme. The required amount of plasmid suspension, usually 1-4µl, was placed into a 0.5ml microfuge tube, along with 1µl of 10x restriction buffer and 1µl of enzyme. The volume was made up to 10µl using ddw. The tube was then placed into a 37°C water bath for 1-2 hours for the digestion to occur. If a larger quantity of plasmid was being digested, volumes of the reagents used were increased accordingly.

For double digests, using two restriction enzymes, 0.5µl of each enzyme was used. If the two enzymes required different restriction buffers, the manufacturer's

guidelines were consulted to determine which buffer to use to obtain maximum performance from both enzymes.

3.2.5 Minigel DNA electrophoresis

Minigels were used to examine the products of restriction enzyme digests quickly and easily. A 0.8% agarose gel was made up in 1x TBE (see appendix I), with the addition of 5µl 10mg/ml ethidium bromide (Sigma) per 100ml of gel. A BioRad Wide Mini-Sub® gel GT system, connected to an EPS 300 power supply (Amersham Pharmacia), was used to separate the restriction products. 2µl of electrophoresis loading buffer (see appendix I) was added to each sample before loading. Gels were run in 1x TBE buffer, and a molecular weight marker appropriate to the expected size of the fragments was included, for example λ/*Hind*III (Life Technologies). After the blue loading dye had reached an appropriate distance along the gel, the gel was removed from the tank and examined under ultraviolet light to reveal the positions of the ethidium bromide-stained bands. A photograph could then be taken to record the image.

3.2.6 Extraction of DNA from an agarose gel (GlassMax method)

The required amount of plasmid DNA was digested and electrophoresed on a minigel as described above. The band containing the restriction product of interest was excised from the agarose on a UV light box. Extraction of DNA was performed using a GlassMax DNA Isolation Spin Cartridge System (Life Technologies), according to the manufacturer's instructions. 0.45ml NaI binding solution per 0.1g gel was added to the agarose piece in a 1.5ml microfuge tube, along with 20µl TBE-enhancing buffer per 0.1g gel. The microfuge tube was then placed in a 50°C water bath for approximately 5-7 min, until the agarose dissolved. The solution containing the dissolved agarose and DNA was placed into a spin column/ collection tube assembly, and centrifuged for 20 sec at 13,000rpm. 0.4ml wash buffer, at 4°C, was then placed into the column, and centrifuged for 20 sec, as before. This step was repeated twice more, with the collection tube being emptied after each spin. After

centrifuging at 13,000rpm for 1min, to remove any excess wash buffer, the spin column was removed to a sample recovery tube, and 40µl of TE buffer at 65°C was added. The column was again centrifuged at 13,000rpm for 20sec, during which time the DNA was eluted with the TE buffer into the recovery tube.

3.3 DNA analysis

3.3.1 DNA preparation from ES cell pellets

ES cell pellets for DNA extraction were prepared as described in section 3.1.7. 750µl of DNA lysis buffer (see appendix I) was added to each pellet, along with 40µl of 20mg/ml proteinase K. This was incubated overnight at 37°C. 750µl of phenol was then added to each tube, and mixed thoroughly. Tubes were then microcentrifuged at 13,000rpm for 2min, then the upper, aqueous, phase was immediately removed into a fresh tube, and the remaining phase discarded. This process was then repeated with a further 750µl of phenol, and then 750µl of chloroform. The aqueous phase was removed after the chloroform stage into a fresh tube, as before, then the DNA was precipitated using an equal volume of isopropanol, and then microcentrifuged at 13,000rpm for 10min to obtain a DNA pellet. The supernatant was then removed, and the pellets left for approximately 15min to air dry. Pellets were resuspended in 20-100µl TE buffer, then stored at 4°C.

3.3.2 Restriction enzyme digestion of genomic DNA

Restriction enzyme digestion of genomic DNA was performed using *Hind*III (Life Technologies) with the supplied REact II buffer. Digestions were performed in a 50µl volume, using 5µl of 10X buffer, 4µl of enzyme, 1µl of 50mM spermidine (Sigma) and the required volume of DNA suspension. The volume was made up to 50µl using deionised distilled water (ddw). The reagents were combined in a 1.5ml microfuge tube, then incubated in a 37°C water bath overnight. Agarose gel electrophoresis was then performed on the digested samples, as described in section 3.3.3.

3.3.3 Agarose gel electrophoresis of DNA

DNA electrophoresis was performed using a 0.7% agarose gel, made up in 1x TBE buffer, with 5µl of 10mg/ml ethidium bromide per 100ml of gel in a horizontal electrophoresis tank (H4 SET, Anachem). 10 µl of loading buffer was added to each sample before loading on to the gel. 30µl of λ/*Hind*III size marker were run alongside the samples for sizing reference. The electrophoresis tank was connected to a power supply, and the gel run at 40-50mA overnight, or until the dye front approached the end of the gel. The gel tray was then removed from the electrophoresis tank, and photographed under ultraviolet light.

3.3.4 Southern transfer

The gel was then removed from the gel tray, placed in denaturing solution (see appendix I) and rocked on a 3D rocking platform for 30-40min. Southern transfer was then carried out using a Blot Transfer System (Life Technologies), in denaturing solution with Whatman 3MM paper as a wick. A Hybond filter was then soaked in denaturing solution and placed on top of the gel, followed by two further pieces of 3MM paper, which had been cut to the size of the gel and filter. Two approximately 20cm high stacks of green paper towels were placed side by side on the top, covering the whole surface of the pile. The DNA was left to transfer overnight, after which time the filter was removed and placed inside a 3MM paper folder to dry for approximately an hour. The filter was then placed in a plastic bag and stored at room temperature until required.

3.3.5 Radio-labelling of DNA probe

Radioactive labelling of probes was performed using a Stratagene RmT probe labelling kit. A tube of reaction mixture was used for each probe to be labelled. To this tube was added 25-50ng of probe DNA, and ddw to a final volume of 42µl. The reaction tube was then placed in a boiling water bath for 5min, then 3µl of magenta

polymerase was then added. 5µl (50 µCi) of α-^{32}P dCTP (ICN) were added to each tube in a safety cabinet, behind a perspex shield. Tubes containing radioactive material were always handled with forceps, behind shields. The reaction tubes were then placed in a shielded water bath at 37°C for 1½- 2 hours.

Unincorporated ^{32}P was removed from the labelled probe using a Nick translation column (Amersham Pharmacia). The reaction tube was removed from the water bath and 20µl of 10mg/ml salmon sperm DNA was added. The Nick column was clamped above a fresh 1.5ml microfuge tube, and the entire 70µl contents of the reaction tube were added, followed by 400µl of TE. When this had run through, the collection tube was replaced with a screw-capped 1.5ml tube. A further 400µl of TE was added to the column. The resulting run-through contained the labelled probe. The amount of radioactivity present was compared with that retained in the column, using a Geiger counter, and the probe only used to label a filter if the reading from the eluate was greater that that from the column. The labelled probe was either used immediately to label a filter, or stored in a lead pot at -20°C until required.

3.3.6 Hybridisation of labelled probe to filter

The filter was dampened with 2x SSC (see appendix I), rolled up with the gauze from a Hybaid hybridisation bottle, then placed into the bottle. 0.5ml of 10mg/ml sonicated salmon sperm DNA was placed into a microfuge tube, and heated in a boiling water bath for 5min, before being added to 25 ml of hybridisation solution (see appendix I), which had been prewarmed to 65°C. The resulting hybridisation mix was added to the hybridisation bottle with the filter, and then left to prehybridise while rotating in the hybridisation oven at 65°C for 3 hours. The labelled probe was then heated in a boiling water bath for 5min, then added to the hybridisation solution in the hybridisation bottle. The bottle was then returned to the hybridisation oven and left to rotate at 65°C overnight.

The liquid contents of the hybridisation bottle were flushed away with an excess of water down a drain suitable for the disposal of small amounts of radioactive isotopes. The filter was then rinsed with 2x SSC, before being washed once with

100ml of washing solution (see appendix I) that had been prewarmed to 65°C, at 65°C for 30min. This washing solution was then discarded, and a further 100ml of prewarmed washing solution added to the bottle, which was returned to the hybridisation oven for another 30min. The filter and gauze were removed from the bottle using forceps, and the filter was blotted between two sheets of Whatman 3MM paper, before being wrapped in Saran wrap, placed into an autoradiography cassette, and exposed to Fuji Medical X-ray film at -70°C for 24 hours. The film was then removed and developed using a hyperprocessor (Amersham Pharmacia), and the strength of the signal obtained used to determine the length of any further exposures, if necessary.

3.3.7 Polymerase chain reaction

PCR reactions were performed in 0.5ml microfuge tubes using a Hybaid Omnigene thermal cycler. The reaction consisted of 1µl of 1:1000 dilution of WTV2 plasmid stock, 1mM $MgCl_2$ (Life Technologies), 125µM each primer (Genosys), 200µM each dATP, dCTP, dGTP, dTTP (Pharmacia Biotech), 1 unit of *Taq* DNA polymerase (Life Technologies) and 1x PCR buffer (Life Technologies). The volume was made up to 50µl with ddw, then the reaction mixture was overlaid with 50µl of paraffin oil to prevent evaporation.

The PCR reaction was carried out for 30 cycles of amplification under the conditions described in appendix II. 10µl of reaction was then removed and electrophoresed on a 2% agarose minigel (see section 3.2.5), with the addition of 2µl of DNA loading buffer.

3.3.8 DNA sequence analysis

Sequencing of the PCR product was performed using a Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia). 5µl of the WTV2 PCR product was incubated at 37° for 15min in a heating block with 1µl shrimp alkaline phosphatase and 1µl exonuclease I, to clean away elements from the PCR reaction mix, such as

primers and excess dNTPs. The tube was then heated to 80°C in a heating block for 15min to stop the reaction and kept on ice until used.

Two reaction mixtures were then made up, one for each of the forward and reverse primers. These consisted of 1µl of the prepared PCR product, 125µM of the primer, and 2µl of reaction buffer, made up to 20µl with ddw. Eight termination mixtures were made up, one set of each of the 4 ddNTPs for each primer. These consisted of 2µl of dGTP termination master mix plus 0.5µl of the (α-^{33}P)ddNTP (0.45µCi/µl, Amersham). 4.5µl of the reaction mix was added to each termination mix, one set of four for the forward primer mix, one set for the reverse primer mix. The contents of the tubes were mixed by vortexing, then overlaid with 50µl of paraffin oil. The reactions were then placed in the thermal cycler and amplified under the same PCR conditions as were originally used with the WTV2 primers (see appendix II). After the reactions were complete, 4µl of stop mix was added to each tube. The tubes were stored at 4°C until used.

The products of the sequencing reactions were separated using a 6% denaturing polyacrylamide gel. This was carried out using BRL S2 sequencing apparatus (Life Technologies). 65ml of 6% denaturing gel solution (see appendix I) was polymerised by the addition of 700µl 10% ammonium persulphate (Life Technologies) and 75µl TEMED (Sigma). This was immediately poured between the two glass plates, which were laid horizontally, fastened by bulldog clips, and separated by 0.4mm spacers. A shark's tooth comb was inserted into the end of the gel before polymerisation had occurred. After the gel had polymerised, it was fitted into the sequencing apparatus, and 1X TBE buffer placed in the upper and lower reservoirs. The gel was pre-run at 80W for half an hour before loading.

The reaction product was added to 10µl of sequencing gel loading dye (appendix I) and denatured in a heating block at 95°C for 2-3min. The wells were flushed out using running buffer from the upper reservoir before the samples were loaded. The gel was run at 80W for 2 hours, after which the gel was transferred on to a piece of 3MM paper. This was overlaid with Saran wrap and then dried for 2 hours using a vacuum dryer (BioRad). The Saran wrap was then removed and the gel exposed to Kodak Biomax MR-1 film (Sigma) overnight at room temperature.

3.4 RNA analysis

Before commencing RNA work, the bench surface was cleaned using RNase Away reagent (Life Technologies). All pipetting was carried out using filter tips which were racked into clean boxes using clean gloves and autoclaved before use. A separate bag of microfuge tubes was set aside for RNA work, and these were only handled using clean gloves. Solutions for RNA analysis were made up using DEPC-treated water (see appendix I) in glass bottles which had been baked at 180°C for 4 hours, or in sterile disposable tubes. Stocks of reagents such as chloroform, isopropanol and ethanol were set aside for RNA use only. All tubes and reagents were only handled using gloves. Gel tanks were cleaned using RNase Away reagent before each use, as were the flasks in which the gels were made.

3.4.1 Total RNA preparation from ES cell pellets

RNA was extracted from either freshly prepared or quick frozen (see section 3.1.9) ES cell pellets. Pellets had been prepared such that each contained approximately 1 x 10^7 cells. 1ml of TRIzol reagent (Life Technologies) was added to the cell pellet, which was then vortexed to resuspend the cells. At this point, cells which had been stored in liquid nitrogen were transferred into an RNase-free 1.5ml microfuge tube. The cell suspension was then left at room temperature for 5 minutes. 0.2ml chloroform and 0.3ml DEPC-treated water (see appendix I) were then added to each tube, which were each then vortexed for 15 seconds. After 2-3 minutes, the tubes were microfuged at 13,000rpm for 5min. The upper, aqueous, phase was then removed immediately from each tube and placed into a fresh RNase-free 1.5ml tube.

The RNA was then precipitated from the aqueous phase by the addition of 0.5ml isopropanol. After mixing, the tubes were placed at -70°C for a minimum of 1 hour, then centrifuged at 13,000rpm at 2-8°C for 10min to precipitate the RNA. The supernatant was then removed and the pellets washed with 0.4ml 75% ethanol and centrifuged at 13,000rpm at 2-8°C for 5 minutes. The supernatant was removed and

pellets left to air dry for 15min at room temperature before being resuspended in 0.1ml DEPC-treated water by leaving at room temperature for a further 15min.

After samples were taken for analysis of RNA integrity and concentration (section 3.4.2), the RNA was reprecipitated by the addition of 2.5vol 100% ethanol and 1/10 vol 3M sodium acetate, and stored in this form at -70°C.

3.4.2 Measurement of RNA integrity and concentration

2μl of each RNA sample were diluted in 1ml DEPC-treated water for measurement using a spectrophotometer (Cecil CE2020). 0.5ml of this was used for measurement of RNA concentration at a wavelength of 260nm. The remaining 0.5ml was used to measure RNA integrity by comparing absorbance at 260 and 280nm. A ratio of 1.8 to 2.0 is obtained when the RNA is undegraded and protein-free.

The integrity of the RNA was also examined by running 1μl of each sample on an agarose minigel, which should reveal the presence of strong 18 and 28S ribosomal RNA bands if the RNA is intact. A 1.2% agarose gel in 1X TBE buffer was made, containing 5μl of ethidium bromide per 100ml. Samples were made up to 10μl by the addition of 9μl of DEPC-treated water, and 2μl of RNA loading dye (see appendix I) was added. Samples were run at 100V until the 18 and 28S ribosomal RNA bands were clearly separated when viewed using a UV light-box.

3.4.3 PolyA$^+$ RNA preparation from total RNA

Preparation of poly A$^+$ RNA was carried out using a Mini-Oligo(dT) Spin Column Kit (Flowgen). The mini-oligo(dT) cellulose spin column was inverted several times to resuspend the resin, then positioned on top of an RNase-free 1.5ml collection tube with a slotted cap. These were then placed inside a 15ml conical-bottomed centifuge tube, and centrifuged at 200 x g for 10 seconds in a MSE Mistral 1000 to drain the storage buffer from the column. The buffer in the collection tube was then discarded, and the column returned to the collection tube.

The total RNA sample was precipitated by centrifuging at 4°C for 30min, then washed in 400μl 75% ethanol before being resuspended in 1ml DEPC-treated

water, to achieve a concentration of 1mg/ml. The sample was then heated to 65°C for 5min before 0.25ml of the supplied loading buffer was added and the sample cooled on ice.

1ml of supplied 0.5M NaCl buffer was applied to the spin column, which was then uncapped and replaced on the 1.5ml collection tube, and centrifuged at 200 x g for 10 seconds. The buffer was discarded from the collection tube, then the RNA sample applied to the column, which was recapped and inverted to mix the sample with the oligo(dT) cellulose resin. The column was then incubated at room temperature for 15min, with mixing every 2-3min. The column was replaced on the collection tube and centrifuged at 200 x g for 10sec. The buffer was discarded from the collection tube, then 1ml of 0.5M NaCl buffer was applied to the column, which was centrifuged at 200 x g for 10sec. The buffer was discarded from the collection tube, as before, then 1ml of supplied 0.1M NaCl buffer was applied to the column, which was centrifuged at 200 x g for 10 sec. The buffer was discarded, and a second 1ml of 1.0M NaCl buffer was applied, and the column centrifuged as before. The column was then removed to a fresh 1.5ml slotted cap collection tube. 0.5ml of supplied elution buffer, which had been prewarmed to 65°C, was applied to the tube, which was immediately centrifuged as before. To elute the remainder of the polyA$^+$ RNA, a further 0.5ml of prewarmed elution buffer was applied, and the column immediately centrifuged again.

The eluted polyA$^+$ RNA was then precipitated by the addition of 20µl of 1mg/ml mussel glycogen, 100µl of 3M sodium acetate (pH 5.2) and 2.5ml (2.5 volumes) of cold 95% ethanol. The samples were mixed well, then placed at -70°C for at least 2 hours to precipitate the RNA. The precipitate was collected by centrifugation at 13,000rpm for 30min at 4°C. The pellet was washed twice with cold 70% ethanol, then once with cold 95% ethanol and left to dry before being resuspended in 30µl of DEPC-treated water.

3.4.4 Agarose gel electrophoresis of RNA

An aliquot containing the required amount (10-30μg) of RNA ethanol precipitate was centrifuged at 13,000rpm for 30min at 4°C. The resulting pellet was then washed in 100μl of 75% ethanol and centrifuged at 13,000rpm for 5min at 4°C. The supernatant was again removed, and the pellet left to dry on ice. When dry, the pellet was resuspended in 30μl of RNA sample buffer (see appendix I) and left on ice to resuspend. 3μl of RNA molecular weight marker (0.24-9.5kb RNA ladder – Life Technologies) was run with the samples. This was made up to 30μl by the addition of RNA sample buffer, then treated the same as the other samples.

The RNA gel (see appendix I) was made by boiling together the agarose, 10x MOPS buffer (see appendix I) and ddw to dissolve the agarose. The gel was then cooled to 60°C in a water bath, after which the formaldehyde was added in a fume hood, and the gel poured in the hood, where it was left to set. The gel tank, tray, combs and gel ends were all treated with RNase Away, as before, to remove any RNase contamination.

3μl of RNA loading dye (see appendix I) and 1μl of 10mg/ml ethidium bromide were then added, and the sample was denatured in a water bath at 65°C for 5min, before being chilled on ice and loaded onto the RNA gel. The gel was run in the fume hood using 1x MOPS buffer at 30mA overnight.

3.4.5 Northern transfer

After being photographed under UV light, the RNA was transferred from the gel to a membrane by Northern transfer. This was carried out using a Blot Transfer System (Life Technologies) as described in section 3.3.4, using RNA transfer buffer (see appendix I), on to a Zeta-Probe® nylon blotting membrane (BioRad). After the transfer, the membrane was soaked in 2x SSC, then left to dry between two sheets of 3MM paper. It was then viewed under UV light, and the positions of the RNA marker bands and the 18 and 28S ribosomal RNA bands marked on the membrane using a ball-point pen. The membrane was then placed between two sheets of 3MM

paper and baked in an oven at 65°C for approximately 2 hours. It was then stored at 4°C in a plastic bag until use.

3.4.6 Radio-labelling of cDNA probe

Probes for hybridisation to Northern blots were prepared in the same manner as those for the analysis of Southern blots, as described in section 3.3.5.

3.4.7 Hybridisation of labelled probe to filter

The filter to be hybridised and the gauze were rolled together and placed into the hybridisation bottle, as described in section 3.3.6. Hybridisation of Northern blots was then carried out using ExpressHyb™ hybridisation solution (Clontech). 10ml of ExpressHyb™ solution, which had been prewarmed to 65°C was added to the hybridisation bottle, along with 100µl of 10mg/ml sonicated salmon sperm DNA, which had been denatured by boiling for 5min. The bottle was placed into the hybridisation oven and rotated at 68°C for 30min. The radioactively-labelled probe was denatured by boiling for 5min, as described for DNA filters, then mixed with the solution from the hybridisation bottle, and the bottle returned to the hybridisation oven to rotate for 1 hour at 68°C.

The radioactive hybridisation solution was removed, then the filter was washed once using 2x SSC. The filter was then washed twice for 20min with 10ml of warmed ExpressHyb™ solution, by returning to the oven and rotating at 68°C. After this, the filter was removed using forceps and was immediately wrapped in Saran wrap. The positions of the markers and the 18 and 28S rRNA bands were marked using an ordinary lab marker, into which had been added a small amount of ^{33}P-αdATP. This renders the marker positions visible on the autoradiograph. The labelled filter was exposed to a piece of Fuji medical X-ray photographic film at -70°C for 24 hours.

After use, the blot could be stripped by incubating in 0.5% SDS in a 95°C water bath with constant agitation for 10min. After being left to cool at room

temperature for 10min, the filter was blotted dry and stored in a plastic bag at -20°C until required.

3.4.8 DNaseI treatment of total RNA

Total RNA to be used for reverse transcription into cDNA was first treated with DNaseI to remove any contaminating genomic DNA. Before treatment, the RNA was diluted to a concentration of 1mg/ml. 0.5ml of RNA was incubated with 100µl 10x DNaseI buffer (see appendix I), 5µl DnaseI (10 units/µl; Roche) and 395µl DEPC-treated water at 37°C in an oven for 1 hour.

100µl of 10X termination mix (see appendix I) was then added, followed by an equal volume (1.1ml) of phenol: chloroform: isoamyl alcohol (25:24:1; v:v:v) (Sigma) and vortexed thoroughly. Each tube was then spun in a microfuge at 13,000rpm for 10min to separate the phases. The top aqueous layer was then transferred to a clean RNase-free tube and the lower phases were discarded. This process was repeated with a further 1.1ml of phenol: chloroform: isoamyl alcohol. 1.1ml of chloroform: isoamyl alcohol (24:1; v:v) (Sigma) was then added to the aqueous phase and mixed thoroughly. Each tube was spun in a microfuge at 13,000rpm for 10min to separate the phases. The aqueous layer was removed and transferred into a 2ml RNase-free microfuge tube. 1/5 volume of 7.5M NH_4OAc and 2.5 volumes of 96% ethanol were added, and the mixture vortexed thoroughly. The tubes were then microfuged at 13,000rpm for 20min, and the supernatant carefully removed. The pellets were gently washed with 200µl of 80% ethanol, then microfuged at 13,000rpm for 10min. The supernatant was carefully removed, then the pellets left to air-dry for approximately 10min before being resuspended in 500µl of DEPC-treated water.

3.4.9 Reverse transcription of total RNA

First strand cDNA synthesis was carried out using the SuperScript™ Preamplification System (Life Technologies). This was performed using the method suggested by the manufacturers for first strand synthesis using oligo(dT) primers. 1-5

μg of DNaseI-treated total RNA was placed into each of two RNase-free 0.5ml microfuge tubes, along with 1μl of the oligo$(dT)_{12-18}$ provided in the kit and sufficient DEPC-treated water to make the volume up to 12μl. Samples were incubated at 70°C in a heating block for 10min, then placed on ice for at least 1min. To each reaction tube was then added, in the following order: 2μl 10x PCR buffer, 2μl 25mM $MgCl_2$, 1μl 10mM dNTP mix, 2μl 0.1M DTT. The samples were then incubated at 42°C in a heating block for 5 min, before 1μl of SuperScript™ II reverse transcriptase was added to one of each pair of reaction tubes and the samples returned to the 42°C heating block for a further 50min. The reactions were terminated by placing the tubes in a 70°C heating block for 15min, then the samples were chilled on ice. 1μl of RNase H (1-4 units/μl) was added to each tube, and the samples were incubated at 37°C in a heating block for 20min. cDNA was stored at -20°C until required.

3.4.10 RT-PCR analysis of cDNA

PCR reactions were carried out in 0.5ml microfuge tubes, in a reaction volume of 10μl. A Hybaid Omnigene thermal cycler was used, as before. As the PCR was to be as sensitive as possible, to detect low levels of gene expression, α-^{33}PdATP was incorporated into the PCR products, to enable their detection by autoradiography. For the PCR to be quantitative, the log phase of each reaction had to be identified for a given range of template concentrations. This is the region where the strength of signal produced on the autoradiograph is proportional to the amount of starting template. The number of PCR cycles for which this was the case was determined for each primer pair by setting up reactions with 0.5, 1 and 2μl of the same template. The intensity of the bands produced on the autoradiograph was measured using Molecular Analyst software on a BioRad Gel Doc 1000 machine. When the band strength was seen to double in intensity with a doubling in template volume, the log phase was assumed to have been located, and this number of cycles was then used when amplifying all the samples. These could be assumed to be in the log phase also, provided that the intensity of band fell within the range produced by

the control samples. If this was not the case, the amount of template was adjusted to produce a band intensity within the correct range.

The reaction mix consisted of 200μM of each dNTP, 1.5mM $MgCl_2$ (unless otherwise specified in appendix II), 0.5pmol of each primer, 1 unit of *Taq* DNA polymerase, 1X PCR buffer (for suppliers see section 3.3.7), 0.5,1 or 2μl of cDNA and 0.5μl of 1:5 dilution of 10μCi/μl α-^{33}PdATP (ICN). This was overlaid with 50μl of paraffin oil to prevent evaporation. Reactions were carried out under the conditions described in appendix II. After the reactions were complete, the products were kept at 4°C until used.

PCR products were separated on a denaturing 6% polyacrylamide gel, as described in section 3.3.8, as this produced the clearest band on the autoradiograph. This was carried out using BRL S2 sequencing apparatus (Life Technologies). The gel was poured as described, but a 30 well square-toothed comb was used instead of a shark's tooth comb. The gel was prerun at 80W for 30 min, as before. In the meantime, 10μl of sequencing gel loading dye (see appendix I) was added to each of the PCR reaction tubes. The PCR products were placed at 99°C in a heating block for 3-4min before being placed on ice. The current through the gel was switched off, and the wells flushed out using running buffer from the upper reservoir. 8μl of PCR product was placed into each well, and the gel run at 80W for 3 hours. The gel was dried and exposed to film as described in section 3.3.8.

3.4.11 Expression analysis using Clontech mouse atlas expression arrays

Clontech mouse atlas expression arrays were used to examine gene expression in the mutant and wild-type ES cells and their RA-differentiated progeny. Total RNA for probing the mouse atlas expression arrays was first treated with DNase I, using the method described in section 3.4.8, then polyA$^+$ RNA was purified from the total RNA as described in section 3.4.3. 1μl of this polyA$^+$ RNA was then converted into ^{32}P-labelled cDNA according to the manufacturer's instructions.

In a shielded safety cabinet, 2μl of 5x reaction buffer, 1μl of 10x dNTP mix, 3.5μl of (α-^{32}P)dATP (3,000Ci/mmol, 10mCi/ml), 0.5μl of 100mM DTT and 1μl of

MMLV reverse transcriptase (provided in the kit, except for ^{32}P-αdATP - ICN) were combined in an RNase-free 0.5ml microfuge tube for each RNA sample to be labelled. 1μl of the polyA$^+$ RNA samples (contained in 1-2μl) were each combined in 0.5ml microfuge tubes with 10X CDS primer mix (supplied in kit), and sufficient DEPC-treated water to make the volume up to 3μl. This was incubated in a preheated 70°C heating block for 2min. The tubes were then incubated in a 50°C heating block for a further 2min. The previously prepared master mix was then added to each reaction tube in a shielded safety cabinet, and mixed. The reaction tubes were then placed into a 50°C heating block for 20min, then 1μl of 10x termination mix was added to stop the reaction.

Chroma spin-200 DEPC-H_2O columns (provided in kit) were used to purify the labelled cDNA by removing unincorporated labelled nucleotides and small cDNA fragments. The labelled cDNA sample was carefully applied to the centre of the flat surface of the gel bed and left to fully absorb into the resin. 40μl of deionised water was applied to the column and allowed to completely drain out. This was discarded in a radioactive waste container. Four fractions were then collected by transferring the column to a clean 1.5ml tube, applying 100μl of deionised water to the column and allowing it to completely drain out. The two fractions with the highest activity (usually fractions two and three) combined to make up the probe.

15ml of Clontech ExpressHyb™ hybridisation solution (provided in kit) was prewarmed to 68°C, then 150μl of 10mg/ml sheared salmon testes DNA (Sigma) which had been denatured in a boiling water bath for 5 min was added. The Atlas array was dampened with deionised water, rolled up with a gauze, then placed into a hybridisation bottle. 10ml of the prewarmed ExpressHyb™ solution containing denatured salmon testes DNA was added, and the bottle placed into a hybridisation oven at 68°C and prehybridised on a low speed setting for 30min.

The labelled cDNA probe was mixed with 1/10th volume (about 22μl) of 10X denaturing solution (1M NaOH, 10mM EDTA) in a shielded fume hood, then incubated at 68°C for 20min. 5μl of C_0t-1 DNA (provided in kit) and an equal volume (about 225μl) of 2X neutralising solution (1M NaH_2PO_4 (pH 7.0)) were then added, then the probe was returned to the 68°C water bath for a further 10min. This

was then added to the remaining 5ml of prewarmed hybridisation mix in a shielded fume hood and mixed by inversion. The prehybridisation solution was discarded and replaced with the solution containing the probe. The hybridisation bottle was replaced in the hybridisation oven and left to prehybridise overnight at 68°C.

Washing solutions 1 (2X SSC, 1% SDS) and 2 (0.1X SSC, 0.5% SDS) were prewarmed to 68°C. The hybridisation solution was discarded and replaced with 200ml of prewarmed washing solution 1. The filter was washed at 68°C for 20min at a high speed setting. This was repeated 3 more times. Two additional 20min washes were performed using washing solution 2 at 68°C. The Atlas array was then removed from the bottle using forceps and immediately wrapped in Saran wrap, then mounted on Whatman 3MM paper and either exposed to x-ray film in an autoradiography cassette at -70°C or to a phosphorimager screen at room temperature.

To strip the Atlas array for re-use, the plastic wrap was removed from the array, which was then immediately immersed in boiling 0.5% SDS. This was left to boil for 5-10min, then removed from the heat and allowed to cool for 10min. The array was then removed from the solution and checked for radioactivity using a Geiger counter. If radioactivity could still be detected, the stripping procedure was repeated. Otherwise, the array was wrapped in plastic wrap and stored at -20°C until the next use.

3.5 Protein analysis

3.5.1 Protein preparation from ES cell pellets

Protein was prepared from either fresh ES cell pellets, or from pellets stored at -70°C. 2.5 volumes of sucrose buffer (see appendix I) were used to resuspend each pellet. 1/10 vol. of 1% NP40 was then added to each pellet, before placing them on ice for 5min.

To prepare only total protein from the cell pellet (performed when making positive control protein from M15 cells), 1/10 vol. 5M NaCl was then added. Pellets were left at 4°C for 10min, and then immediately sonicated, using a MSE sonicator.

This was carried out on ice, at ¾ power for 20-30sec. Protein concentration was determined (described below), and aliquots frozen at -70°C.

To prepare both nuclear and cytoplasmic protein (for experimental samples), after 5min on ice, cell nuclei were pelleted by a 10sec microfuge at 13,000rpm, at 4°C. The supernatant, containing the cytoplasmic proteins, was removed and retained. Protein concentration was determined (described below), and aliquots were frozen at -70°C. The pelleted cell nuclei were then washed with 1.5ml PBS at 4°C, then resuspended in 2.5 vol. sucrose buffer, and 1/10 vol. 5M NaCl was added. After being left at 4°C for 10min, sonication was performed, as described above.

Measurement of protein concentration was carried out using a Cecil CE2020 spectrophotometer. Measurements were performed at 595nm in a plastic disposable cuvette. A standard curve was first prepared by measuring the OD of known concentrations of BSA in 1ml BioRad Protein Assay Dye Concentrate, diluted 1:5 with ddw. ODs of the experimental samples were measured, usually at two different dilutions (2µl and 4µl of sample, in 1ml BioRad reagent), and the concentrations of the samples determined using the standard curve. Protein was stored in aliquots at -70°C.

3.5.2 SDS-PAGE

SDS polyacrylamide gel electrophoresis was performed in a Hoefer® SE 600-15-1.0 dual cooled vertical slab unit (Amersham Pharmacia). Gels were prepared as described in the manufacturer's instructions. Two clean 18x16cm glass plates were clamped together, using 1mm spacers. The plates were then inserted vertically into the casting cradle, and fastened into place. A 10% or 8% resolving gel (see appendix I) was prepared first, and poured to a depth of approximately 10 cm. This was immediately overlaid with ddw, to a depth of approximately 0.5cm. After 30min, the gel was set and the water could be poured off. The comb was then placed into the gap between the two glass plates. The stacking gel (see appendix I) was then prepared and poured to level with the top of the glass plates. This was also left to set for 30min before the comb was carefully removed, and the plates unfastened from the casting

cradle and fastened to the upper buffer reservoir. Sufficient TGS running buffer (see appendix I) to cover the bottom of the gel sandwich was placed into the gel tank, and the upper reservoir and gel were placed into position. TGS running buffer was placed into the upper reservoir.

Samples were prepared for electrophoresis by making them all up to the same volume with PBS. A volume of 2x loading buffer (see appendix I) equal to this total sample volume was then added. Samples were heated to 95°C for 3min, just prior to being loaded into the gel. While the samples were being heated, the wells were flushed out using TGS buffer from the reservoir with a 5ml syringe and needle. A Benchmark™ Prestained Protein Ladder (Life Technologies) size standard was also prepared, by taking 20µl of marker and adding the appropriate volumes of PBS and loading buffer. The samples and marker were then loaded into the gel, which was run at 55V overnight.

3.5.3 Western transfer

Transfer of the protein from the gel to a Hybond™- C extra nitrocellulose filter (Amersham Pharmacia) was carried out in a BioRad Trans-Blot™ Cell. The gel and filter were placed between two sheets of 3MM paper and placed inside the transfer insert. The insert was then placed into the transfer tank, in the correct orientation towards the electrodes. The tank was filled with 2.5L of Western transfer buffer (see appendix I), and run at 50V for 2 hours.

The filter was then stained for the presence of protein using an Instaview Nitrocellulose Staining Kit (Merck), according to the manufacturer's instructions. To 97ml of ddw were added 2ml of 50x stain concentrate and 1ml of 100x enhancer concentrate. The filter was placed in a plastic box, and incubated with the staining solution on a rocking platform for 2-5min. The staining solution was then discarded, and replaced by 99ml ddw to which 1ml enhancer concentrate had been added. This was left to rock for a further 5min. The stained filter was then photographed. 20ml of 10x destain concentrate were added to 180ml ddw, and this was used to wash the filter several times.

3.5.4 Protein detection using antibodies

Before application of antibodies to the filter, blocking was carried out to reduce background signal. This was done by incubating the filter in 5% Marvel powdered milk in TBST (see appendix I) for one hour at room temperature. The blocking solution was then replaced with fresh blocking solution containing the primary antibody. The WT1 N-terminal antibody (N-terminal monoclonal mouse anti-human WT1 M3561, clone 6F-H2, subclass IgG1, kappa - Dako), which shall be referred to as WT1 H2, was used at a concentration of 1/500. The WT1 C-terminal antibody (C-terminal polyclonal rabbit anti-human WT1 sc-192, C-19, IgG - Autogen), which shall be referred to as WT1 C19, was used at a concentration of 1/250. The filter was rocked on a 3D rocking platform in primary antibody solution for 1 hour. The antibody solution was then removed and the filter washed in changes of TBST for 4 x 10min (WT1 H2) or 3 x 10min (WT1 C19), with agitation. The secondary antibody was then added in 5% Marvel/TBST. HRP-conjugated sheep anti-mouse IgG (Scottish Antibody Production Unit (SAPU)) was used with WT1 H2, at a concentration of 1/250. HRP-conjugated donkey anti-rabbit IgG (SAPU) was used with WT1 C19, at a concentration of 1/1000. The filter was rocked in secondary antibody solution for one hour. The antibody solution was then removed, and the filter washed in changes of TBST for 4 x 10min (WT1 H2) or 3 x 10min (WT1 C19).

After the washing was completed, the filter was placed into a folder made from two acetate sheets. The folder was then placed into an autoradiography cassette. Detection was performed using the ECL detection system (Amersham Pharmacia). Equal amounts of ECL reagents 1 and 2 were placed in a plastic 20ml universal tube. 0.5ml of each reagent was found to be sufficient for each filter. In the dark room, the mixed ECL reagents were placed into the acetate folder, on to the filter. The folder was closed to prevent contact between liquid and film. After approximately 1min, a piece of ECL film was placed on top of the folder, under safe-light conditions, and the autoradiography cassette was closed. The first piece of film was exposed to the filter for 30 seconds. This was followed by another piece of film for 1min. These two pieces of film were developed in the hyperprocessor, and the level of signal obtained examined. According to this level, further pieces of film were exposed to the filter for

longer or shorter time periods, as necessary, until the required level of signal was obtained.

3.5.5 Immunofluorescence analysis

ES cells for immunofluorescence analysis were grown on 22x22mm glass coverslips, each in a single well of a six-well plate. After aspiration of the cell culture medium, the cells were washed twice in ice-cold PBS, before being fixed with 5ml of 1:1 (v:v) methanol:acetone for 10min at room temperature. The fix was then removed and 5ml of immunofluorescence blocking solution (see appendix I) was added, and the cells left at room temperature to block overnight.

The blocking solution was then removed, and the surface of the petri dish around the coverslip dried to prevent the antibody solution from running off. 100µl of primary antibody (1:250 in blocking solution) was then carefully placed onto the coverslip and left for an hour at room temperature. The cells were then washed three times in PBS/0.05% Tween 20 at room temperature. The inside of the dish around the coverslip was again dried, then 100µl of FITC-conjugated secondary antibody (1:100 in blocking solution) was carefully placed on the coverslip. The coverslips were then left for 30 minutes in the dark at room temperature, before being washed three times in PBS/0.05% Tween 20 at room temperature.

The coverslips were mounted on glass slides using Vectashield (Vector Laboratories) mounting medium, containing 1.5µg/ml DAPI. After sealing the edges of the coverslips using nail varnish (or vulcanising solution if the slides were to be examined using a confocal microscope), the slides were stored in the dark at 4°C.

3.6 *In vivo* methods

3.6.1 Production of chimaeras by blastocyst injection

Blastocyst injections were performed by Jennifer Doig, Tracy Higgins and Charles Patek. Targeted ES cells were injected into day 3.5 blastocysts, F_2(C57Bl/6 x CBA). Only a small number of cells (approximately 2-10) were injected into each

blastocyst. The blastocysts were then implanted into the uterus of a day 2.5 pseudopregnant recipient F_1(C57Bl/6 x CBA).

3.6.2 Glucose phosphate isomerase analysis

This analysis was kindly performed on my behalf by Deborah Bruce. As the ES cells used for injection express GPI 1A and the blastocysts express GPI 1B, the level of chimaerism in any possible chimaeras born could be measured by determining the proportion of each GPI isomer in particular tissues. Method adapted from West and Green, 1983; West *et al.*, 1986. The cellulose acetate plates (Titan III, Helena Biosciences) for running the protein were presoaked in Supre heme buffer (Helena Biosciences) for at least 30 min. Approximately 4mm^2 of each of the tissue samples to be tested, along with standards A and B (mouse foetal liver, A from strains 129/Ola or BALB/c, B from CBA or C57Bl), were homogenised with 2 volumes of sample buffer (see appendix I) in microfuge tubes. Samples were then microfuged at 13,000rpm for 4min and placed on ice until required. The electrophoresis tank was prepared by placing 50ml of heme buffer into the reservoir at each end and placing cooling sponges (kept at -20°C) in the centre. 8µl of the supernatant from each sample was placed into a well on the applicator tray, along with supernatant from standards A and B. The presoaked cellulose acetate plates were blotted dry then placed gel side up on the loading tile. Samples were transferred on to the plate using the applicator. The plates were then placed into the electophoresis tank gel side down and 350V was applied across then for approximately 2 hours.

In the meantime, the agar was prepared by melting on a heating block and keeping at 65°C in a water bath. 7.2ml of agar was transferred into a 20ml glass jar and returned to the water bath. Just before the plates were removed from the electrophoresis tank, 1.2ml 0.5M Tris, 3ml Assay stock (see appendix I), 45µl Glucose-6-phosphate dehydrogenase (G-6-PD) enzyme (1mg/ml)(Sigma), 1.5ml 0.02M 3-[4,5-Dimethylthiazol-2-oyl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and 1.5ml 8mM Phenazine methosulphate (PMS) (Sigma) were mixed in a 20ml universal tube. This was then added to the 7.2ml of warmed agar, mixed and

quickly poured over the gel side of the cellulose acetate plate. The plate was placed in the dark until bands developed revealing the positions of the GPI isomers, then fixed overnight in fixing solution (see appendix I).

3.6.3 Teratoma production

These procedures were carried out by Dr. Colin Miles of the MRC Human Genetics Unit, Edinburgh. The ES cells for injection were removed from their flask as described in section 3.1.2, then washed once with PBS. The cell concentration was measured using an Improved Neubauer haemacytometer. The cells were then centrifuged at 1000rpm in a hanging bucket centrifuge for 5 minutes, the supernatant was removed, then the cells were resuspended in PBS so that the number of cells required for the injection was contained in 0.1ml. 1×10^6-1×10^7 ES cells were injected subcutaneously into the flank of a 129 Ola mouse. Comparable numbers of cells were injected for each genotype on a given day. ES cells of different genotypes could be injected into the same mouse, one genotype into the left flank, one into the right.

Teratomas were left to develop for 25-60 days, with their sizes monitored so that teratomas were not allowed to reach a size at which they were causing the animal distress. Mice were killed and the teratoma mass dissected out and fixed overnight in buffered formalin. Teratomas were then weighed using a top pan balance, and their weights recorded before being placed in 70% ethanol and sent for wax embedding. 3μm sections were cut from the wax blocks using a microtome, which were mounted on glass microscope slides and stained using haematoxylin and eosin. Histology was examined using bright field microscopy.

4. Sequential targeting and analysis of sequentially targeted cells

4.1 Sequential targeting

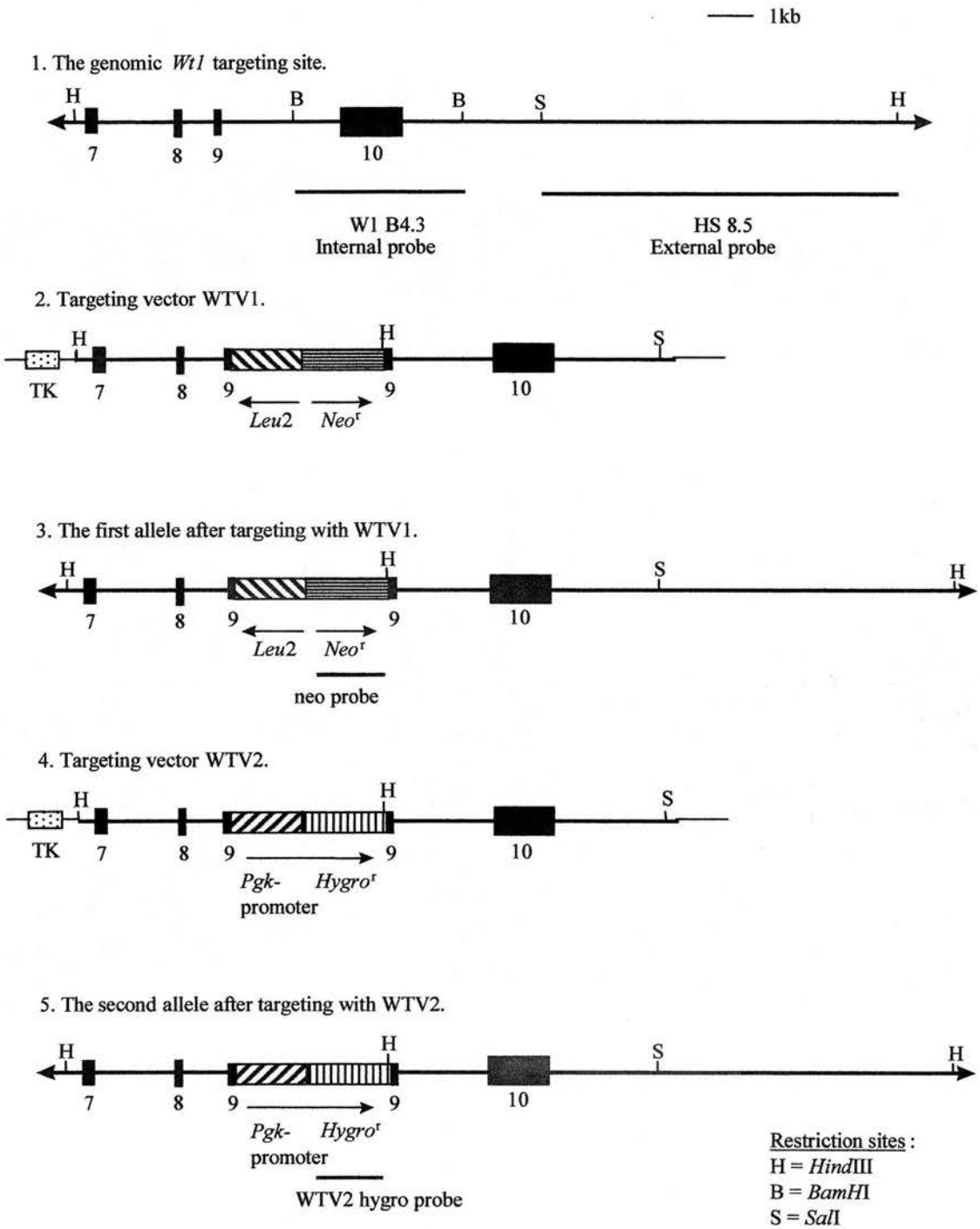
The main aim of the project involved the production and analysis of sequentially targeted ES cells with similar mutations in both alleles of the *Wt1* gene. These mutations would resemble a subset of those found in Denys-Drash syndrome (DDS) (see section 1.6), and would lead to the production of truncated WT1 proteins, lacking the final two of the four WT1 zinc fingers.

The work began using DDS heterozygous CGR8 ES cells, which had previously had one allele of *Wt1* disrupted using the isogenic targeting vector WTV1 (see figure 4.1) (Patek *et al.*, 1999). A second targeting vector, WTV2, similar to the first, but containing a hygromycin rather than G418 selectable marker, had already been prepared for the purpose of targeting the second allele.

4.1.1 Confirmation of genotype of DDS heterozygous ES cells by Southern analysis.

Before introducing the second targeting event to DDS heterozygous ES cells, the genotypes of several heterozygous clones were confirmed using the Wilms' internal probe (W1 B4.3) (figure 4.1). Homologous recombination of the WTV1 targeting vector used to create the initial targeting event leads to the introduction of a novel *Hind*III restriction site, and the reduction of this restriction fragment from 19kb to 13kb (see figure 4.1). All putative heterozygous clones

Figure 4.1 Sequential targeting of *Wt1* using vectors WTV1 and WTV2



analysed produced the anticipated pair of bands at 19 and 13kb after restriction digestion with *Hind*III and agarose gel electrophoresis (figure 4.2, table 4.1).

Table 4.1 Band sizes expected after digestion with *Hind*III

	Expected band size (kb)			
Genotype	**Probe W1 B4.3**	**Probe HS 8.5**	**Neo probe**	**WTV2 hygro probe**
+/+	19	19	-	-
+/-	19 +13	19 + 13	5.7	-
-/-	13	13	5.7	5.8

+/+ wild-type cells

+/- heterozygous cells

-/- compound heterozygous cells

4.1.2 Determination of the optimal concentration of hygromycin B to use to select against non-targeted cells.

Before the selection against untargeted cells using hygromycin could be performed, it was necessary to determine the appropriate concentration of hygromycin to use. The aim was to find a concentration of hygromycin B that would kill cells not possessing the hygromycin resistance sequences present in WTV2, but not be high enough to select for genetic changes, as utilised in high G418 selection

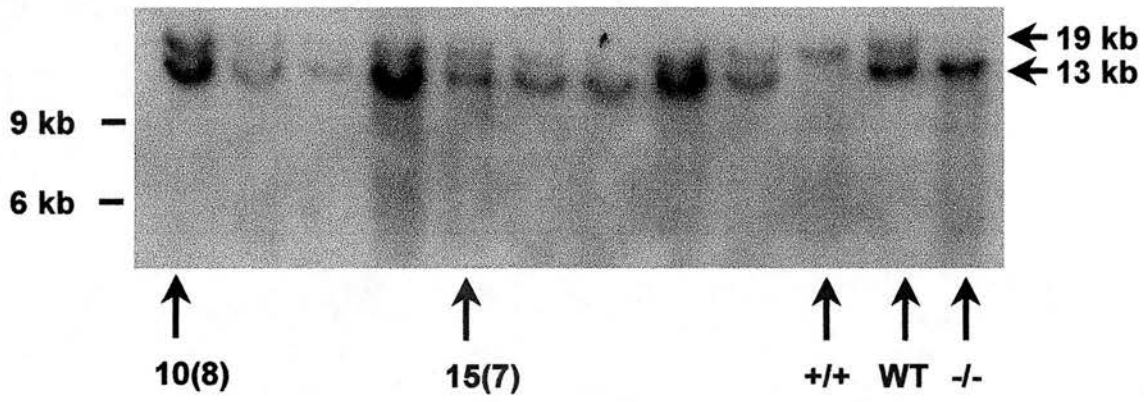


Figure 4.2 Confirmation of genotype of heterozygous clones.

Before introduction of the second targeting event, the genotypes of several clones were determined to ensure that the electroporation was being performed on clones which were heterozygous at this locus. The probe used was the Wilms' internal probe (W1B4.3) (see figure 4.1), which detects an alteration in a *Hind*III restriction fragment from 19 to 13kb. The two heterozygous clones indicated by arrows were the ones later selected for electroporation with vector WTV2.

10(8) = clone 10 at passage 8

15(7) = clone 15 at passage 7

+/+ = DNA from wild-type mouse (129 Ola)

WT = heterozygous DNA from Wilms' tumour found in DDS chimaeric mouse (Patek *et al*, 1999)

-/- = DNA from ES cell made homozygous by high G418 selection (DK3A – Patek *et al*, 1999)

(Mortensen *et al.*, 1992). Heterozygous ES cells were grown until approximately 50% confluent and then various concentrations of hygromycin were added. Hygromycin was added to the cells every two days, as the medium was changed. A concentration was sought at which the cells would die gradually over a period of approximately a week, rather than immediately. Cell survival of the two different heterozygous clones used is shown in table 4.2. Survival of cells was measured on a scale from ++, which indicates that the majority of the cells survived, to -, which represents no surviving cells.

Table 4.2 Determination of concentration of hygromycin to use in selection

	After 5 days of hygromycin		After 12 days of hygromycin	
Conc (μg/ml)	clone 8(8)	clone 15(9)	clone 8(8)	clone 15(9)
50	++	++	+	+
100	+	+	-	-
150	-	+	-	-
200	-	-	-	-
250	-	-	-	-

It was therefore decided to use the hygromycin B at a concentration of 100μg/ml.

4.1.3 Confirmation of chromosome number in heterozygous clones by production of metaphase spreads.

Heterozygous clones which were candidates for sequential targeting had their modal chromosome number per cell checked, to ensure that only clones with the

correct chromosome complement (n = 40) were used to produce compound heterozygous cells.

The two clones selected for electroporation with the second targeting vector (WTV2) were those with the highest proportion of metaphase spreads seen to contain 40 chromosomes. These were clone 15 (passage 7) and clone 10 (passage 8), which contained 75% and 80% (15 and 16/20) metaphases with the correct chromosome count, respectively.

4.1.4 Confirmation of identity of WTV2 plasmid using diagnostic restriction enzyme digestion.

Before introducing the WTV2 targeting vector (figures 4.1 & 4.3) into the chosen heterozygous clones, the identity of the plasmid vector was confirmed by diagnostic restriction enzyme digestion.

Four different restriction enzymes were used to cut the plasmid. The expected sizes of the bands produced with each enzyme were:

a. *Not*I - 16.9kb

b. *Bam*HI - 15.3kb, 1.6kb

c. *Hind*III - 11.1kb, 5.8kb

d. *Eco*RI - 9.0kb, 2.1kb, 2x 1.8kb, 1.4kb, 0.8kb

The plasmid was digested with each of the four enzymes, and bands of the expected sizes were produced in each case (figure 4.4). Thus the plasmid was confirmed as being WTV2, and the electroporation could proceed.

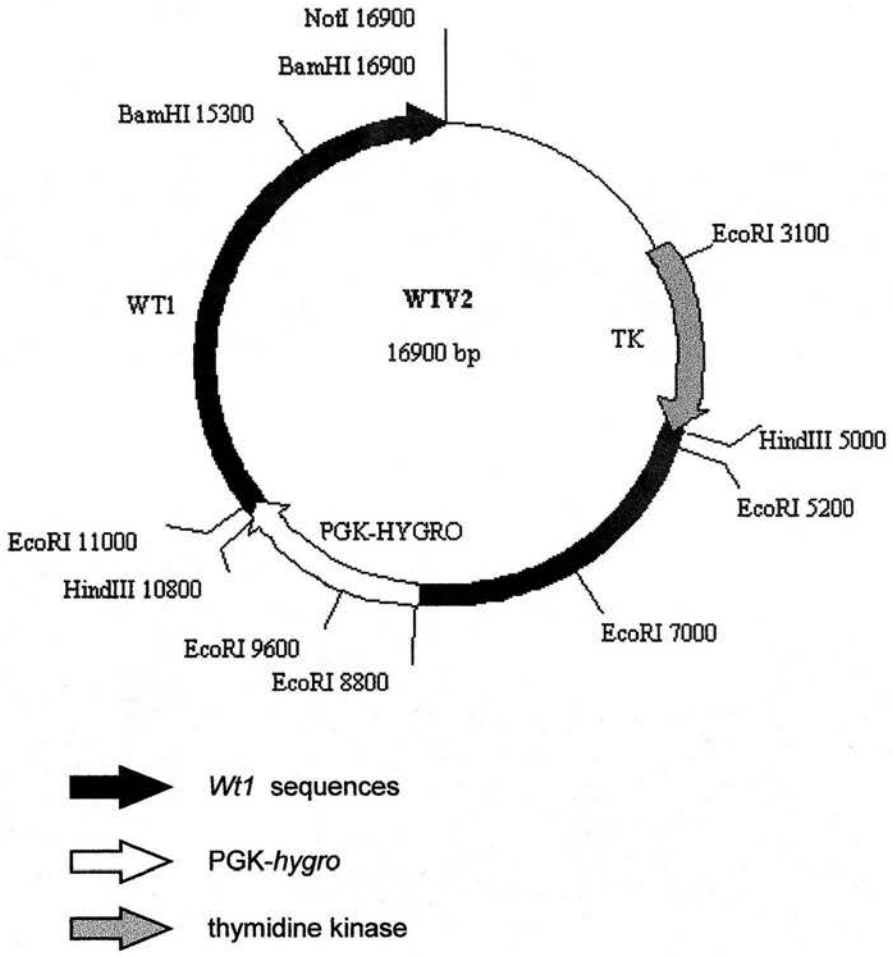


Figure 4.3 Targeting vector WTV2

Vector WTV2 was created for the purpose of targeting the second allele of *Wt1*. The vector contains 9.9kb of sequence homologous to the genomic region containing exons 7, 8, 9 and 10 of *Wt1*. Exon 9 is disrupted by the insertion of the hygromycin B phosphotransferase gene, driven by the PGK promoter sequence. The HSV thymidine kinase (TK) gene is also present, enabling negative selection against clones in which non-homologous recombination has occurred.

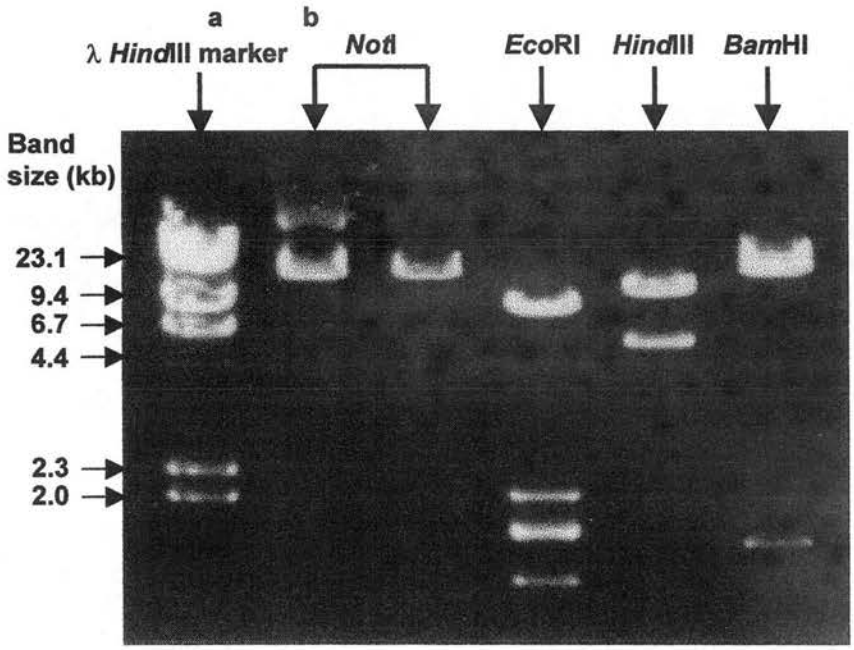


Figure 4.4 Diagnostic restriction enzyme digests to confirm identity of WTV2 plasmid vector.

Approximately 2μg of WTV2 vector was digested with a selection of restriction enzymes which are known to cut the plasmid at specific sites, producing bands of known sizes. All the enzymes used gave bands of the expected sizes, thus confirming the identity of the plasmid and enabling the electroporation to proceed.

*Not*I lane **a** contains twice as much plasmid as lane **b**. The apparently higher molecular weight band is due to residual undigested supercoiled plasmid DNA.

4.2 Analysis of sequentially targeted cells

4.2.1 Southern analysis of clones surviving positive-negative selection

The WTV2 targeting vector was introduced into heterozygous clones 15 (passage 7) and 10 (passage 8) by electroporation. Cells were then grown in the presence of G418 and hygromycin, as correctly targeted colonies should contain regions of both WTV1, which confers G418 resistance, and of WTV2, which confers hygromycin resistance. Selection with both antibiotics prevents survival of cells in which the first targeted allele has been replaced by the second targeting event. After four days, negative selection was also performed by the addition of ganciclovir, which kills colonies in which non-homologous insertion has occurred, via the presence of the thymidine kinase gene (see section 1.1).

Clones surviving the positive-negative selection regimen were picked and cultured, and then DNA was extracted for the purpose of Southern analysis, to confirm the genotype. 167 clones were picked from electroporation A (clone 15, passage 7), and 116 clones were picked from electroporation B (clone 10, passage 8). The first probe used was the Wilms' internal probe (W1 B4.3), which gave only a band of the reduced size, 13kb (see table 4.1), in sequentially targeted cells (figure 4.5). From electroporation A, 25 of 81 clones analysed (28%) gave the desired result, and from electroporation B, 19 from 54 clones (35%) were successful. These clones were then screened using the Wilms' external probe, HS8.5, to confirm that homologous recombination had taken place (figure 4.6). This probe produces the same sizes of mutant and wild-type band as the internal probe. Further screenings were then undertaken using probes for the neomycin resistance gene from WTV1

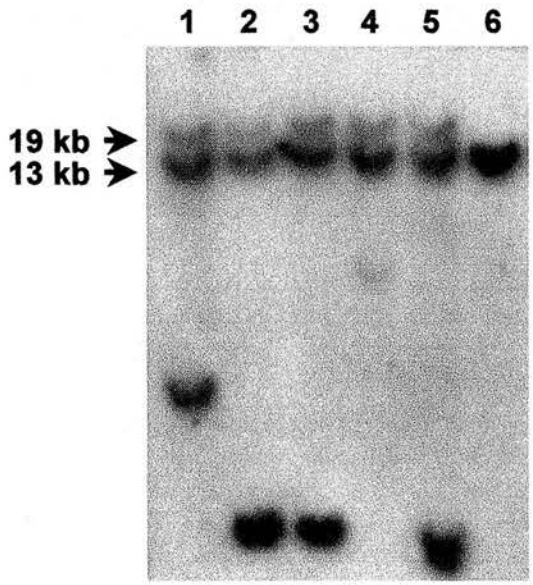


Figure 4.5 Use of Wilms' internal probe (W1B4.3) to identify possible sequentially targeted clones after electroporation with WTV2.

DNA from clones surviving selection with G418, hygromycin B and gancyclovir was digested with *Hind*III. Successfully sequentially targeted clones should reveal only the 13kb band.

Lane 6 shows a clone which was then tested using further probes. The heterozygous clones in lanes 1-5 survived selection with G418 and hygromycin due to random insertions of WTV2 (lower bands).

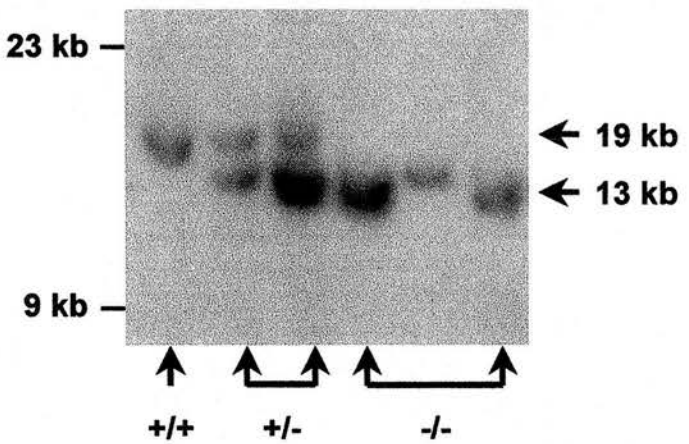


Figure 4.6 Use of Wilms' external probe (HS8.5) to confirm genotypes of sequentially targeted clones.

After use of the Wilms' internal probe to select possible sequentially targeted clones, the Wilms' external probe (see figure 4.1) was used to confirm the genotype. This probe shows the same reduction of a *Hind*III fragment from 19kb to 13kb.

(neo) and the hygromycin resistance gene from WTV2 (WTV2 hygro), to confirm that these sequences were both present in the sequentially targeted clones (figures 4.7 & 4.8, table 4.1 for expected band sizes). All clones that gave the correct band size with the internal probe were shown to have the correct genotype with the other three probes. The high level of success obtained is probably due to the isogenic nature of the targeting vector.

4.2.2 Confirmation of chromosome number in sequentially targeted clones by production of metaphase spreads.

Before any study of the sequentially targeted clones, either *in vivo* or *in vitro*, could be carried out, the chromosome numbers of the clones were checked. Metaphase spreads were again prepared from the clones, and the average number of metaphases containing 40 chromosomes was determined.

All the sequentially targeted clones were seen to have 40 chromosomes in at least 65% of metaphases counted (see table 4.3).

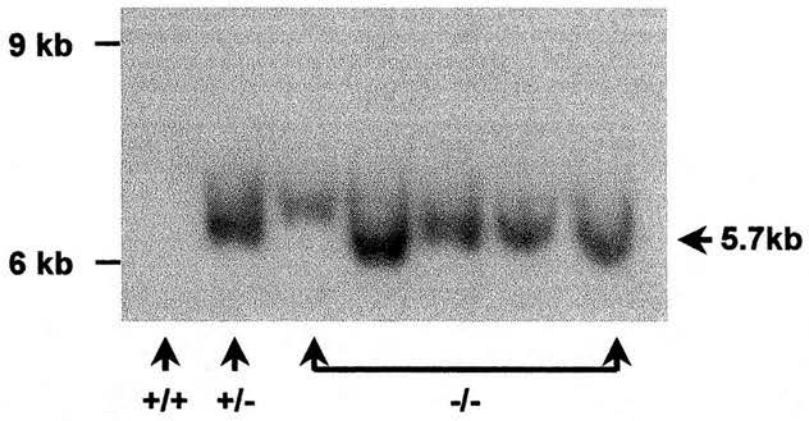


Figure 4.7 Presence of initial targeting event in sequentially targeted clones shown using probe specific to neomycin resistance sequence.

A neomycin resistance probe (neo) was used to confirm the fact that the sequences contributed by the first targeting event were still present in the sequentially targeted clones.

After digestion with *Hind*III, a 5.7 Kb fragment is detected which is not present in wild-type cells.

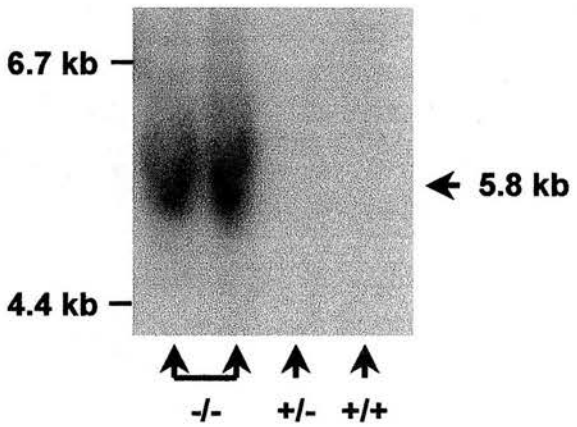


Figure 4.8 Presence of second targeting event in sequentially targeted clones shown using probe specific to hygromycin resistance sequences from vector WTV2.

A probe made from the WTV2 plasmid (WTV2 hygro) was used to confirm the presence of vector sequences in putative sequentially targeted clones. The figure also reveals the absence of these sequences in heterozygous and wild-type cells.

After digestion with *Hind*III, a 5.8kb band was detected in sequentially-targeted clones only.

Table 4.3 Homozygous clones with the proportions of cells seen to contain 40 chromosomes

Electroporation	Clone	Cells with correct chromosome number
A Clone 15, passage 7	37	13/20 (65%)
	39	15/20 (75%)
	46	16/20 (80%)
	47	13/20 (65%)
	51	14/20 (70%)
B Clone 10, passage 8	33	15/20 (75%)
	36	14/20 (70%)
	38	14/20 (70%)
	42	13/20 (65%)

4.2.3 Compound heterozygous nature of sequentially targeted clones revealed by sequencing of vector WTV2.

The section of targeting vector WTV2 containing the *Rsr*II restriction site into which the hygromycin resistance gene was inserted within exon 9 of the *Wt1* gene was sequenced (figures 4.3, 4.9). This was done by PCR amplification of this region, using a forward primer in the *Wt1* exon 9 sequence and a reverse primer in the 5' end of the PGK promoter driving the hygromycin resistance gene (see appendix II for primer sequences). This was to determine the nature of the *Wt1* modification conferred by the targeting event, and compare it with that produced by the first targeted allele.

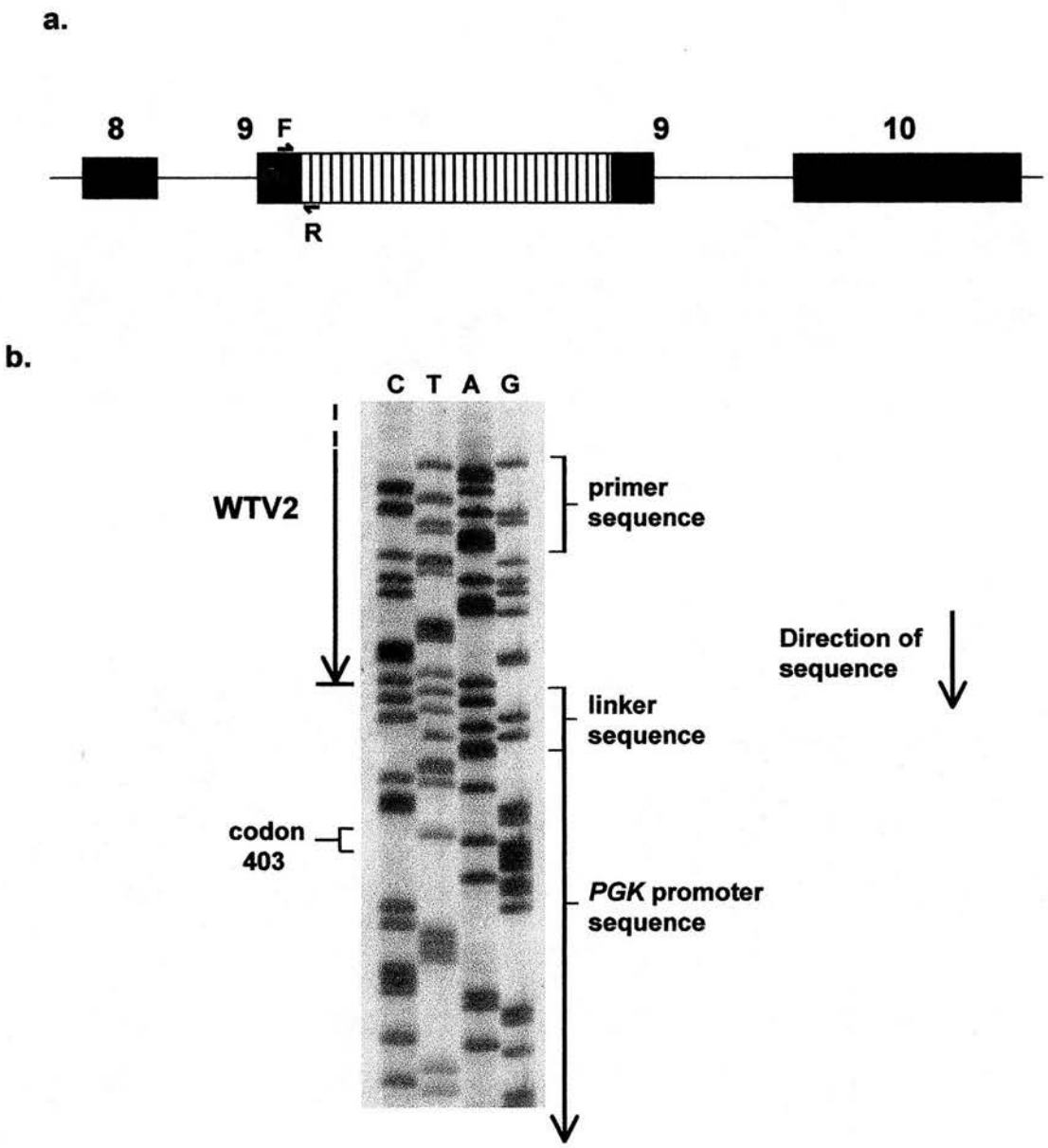


Figure 4.9 Sequencing of targeting vector WTV2 to determine the nature of the modification produced by the second targeting event.

a. Diagram (not to scale) of section of WTV2 plasmid to indicate positions of forward and reverse PCR primers. Numbers indicate exons of *Wt1*. F- forward primer, WTV2F/DS26393 (Patek *et al*, 1999). R- reverse primer, WTV2R.

b. Primers (WTV2F and WTV2R) were designed to produce a PCR product spanning the region where the hygromycin sequences, plus their PGK promoter, were inserted into exon 9 of *Wt1* (see appendix II). This product was then sequenced, revealing the production of a nonsense codon at amino acid position 403 (see figure 4.10).

The sequences of wild-type *Wt1*, the original DDS allele $Wt1^{tmT396}$ and the second allele produced by WTV2 are compared in figure 4.10. When the sequence of this region in vector WTV2 was examined, it was seen to result in the production of a stop codon only 7 amino acids downstream from the position of the stop codon in $Wt1^{tmT396}$. For this reason, the second allele of *Wt1* was named $Wt1^{tmT403}$.

The original modification leads to the truncation of the WT1 protein in ZF3, leading to the functional loss of both ZF 3 and 4 and loss of the KTS region. This means that it can functionally be classed as a Denys-Drash syndrome mutation (see section 1.6). Insertion of WTV1 led to an amino acid substitution (S395R), immediately followed by a translational stop codon at position 396 (Patek *et al.*, 1999). The second modification, produced by targeting with vector WTV2, leads to 8 amino acid substitutions, beginning at position 395, followed by a stop codon at position 403. Although this obviously produces a longer protein than the first targeted allele, the substitutions of the functional amino acids making up this part of ZF 3 with the random sequence produced by the PGK promoter region will cause disruption of the zinc finger at the same place as the protein produced by WTV1 insertion. The amino acids immediately following the point of termination (or amino acid substitution) are some of those which are directly involved in zinc complexing (amino acids 401 and 405) (Borel *et al.*, 1996). Their loss or replacement will completely prevent correct zinc finger formation. The mutations produced by insertion of vectors WTV1 and WTV2 lead to the removal of amino acid 396 in one case (WTV1) and its substitution in the other. This amino acid is involved in DNA sequence recognition (Pavletich and Pabo, 1991) and its loss is sufficient to disrupt WT1, as several DDS patients have been reported with point mutations leading to

Wild-type *Wt1*

394 R S D H L K T H T R

1657 CGG TCC GAC CAT CTG AAG AAC CAC ACC AGG

First mutant allele - *Wt1*^{tmT396}

394 R R *

1657 CGG CGA TAG

Second mutant allele - *Wt1*^{tmT403}

394 R S S S M N S T G *

1657 CGG TCA TCA TCG ATG AAT TCT ACC GGG TAG

* denotes position of stop codon

Figure 4.10 Comparison of the wild-type *Wt1* allele with the mutations produced by the first and second targeting events.

The first mutant allele was generated as described in Patek *et al*, (1999). The second was produced by the introduction of vector WTV2 to the previously targeted cells. Insertion of the PGK-hygro cassette into exon 9 of *Wt1* is seen here to produce a stop codon at amino acid position 403, and is therefore predicted to lead to a protein truncated 7 amino acids downstream from the truncation produced by the first targeting event.

amino acid substitutions at this site (Pelletier *et al.*, 1991b; Baird *et al.*, 1992; Little *et al.*, 1993; Nordenskjold *et al.*, 1994). Neither of these two proteins is therefore predicted to be able to bind to the usual WT1 DNA consensus sequences (Drummond *et al.*, 1994; Hamilton *et al.*, 1995).

4.2.4 Absence of *Wt1* expression in undifferentiated ES cells revealed by Northern and Western analysis.

After production of sequentially targeted DDS cells, and their confirmation as such by Southern analysis, the next step was to characterise the cells in terms of *Wt1* expression, by both Northern and Western analysis. Previous analysis of the DDS heterozygous clones and some homozygous clones produced by high G418 treatment (Mortenson *et al.*, 1992) of the heterozygotes had revealed that *Wt1* expression could be detected in undifferentiated ES cells (Patek *et al.*, 1999). However, study of wild-type, heterozygous and compound heterozygous ES cells in the present study, using both Northern and Western analysis, failed to detect any *Wt1* signal in undifferentiated cells (figure 4.11). As the Western analysis was performed using the same protocol and the same antibody as had been used in the previous work, the failure to detect WT1 protein here is unlikely to be due to experimental differences in sensitivity. It is more likely that the ES cell cultures in which *Wt1* expression had previously been detected contained a higher proportion of differentiated cells than those studied here, as it has been demonstrated that ES cells induced to differentiate by the addition of all-trans retinoic acid exhibit high levels of *Wt1* expression (Scarnhorst *et al.*, 1997).

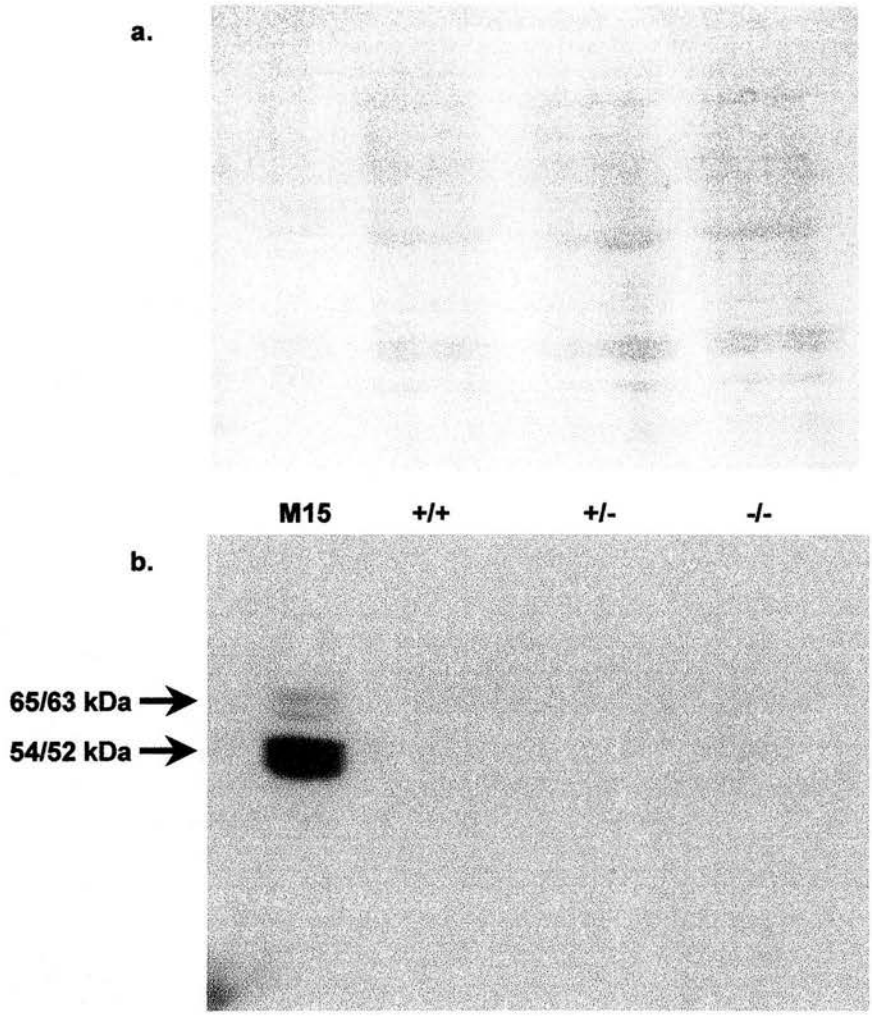


Figure 4.11 Western analysis of nuclear extracts from undifferentiated ES cells.

Nuclear protein was extracted from undifferentiated wild-type, DDS heterozygous and DDS homozygous CGR8 ES cells. 75μg of this was separated using SDS-PAGE on a 10% polyacrylamide gel, along with 10μg of positive control total protein from the M15 mouse mesonephric cell line (Larsson *et al*, 1995). Western analysis was performed.

a. The filter was stained using the Instaview system (BDH) to reveal the relative amounts of protein transferred.

b. The N-terminal antibody 6F-H2 (Dako) was used to detect the presence of WT1 protein.

4.3 Use of retinoic acid to induce *Wt1* expression in ES cells

As undifferentiated ES cells were found to not express detectable *Wt1* RNA or protein, the presence of mutant RNA transcripts and truncated proteins in the targeted cells could not be confirmed. It was particularly important that this was done, as previous work had revealed a discrepancy between levels of mutant and wild-type RNA and protein in DDS heterozygous cells, with the mutant and wild-type transcripts being present at approximately equal levels, yet a 20:1 excess of wild-type over mutant protein detected (Patek *et al.*, 1999). More relevant to the DDS compound heterozygous cells, cells that had been made homozygous for the original mutation using high G418 selection revealed a total absence of WT1 protein (C. Miles, personal communication), despite the presence of *Wt1* mRNA (Patek *et al.*, 1999). Treatment with all-trans retinoic acid has been shown to increase *Wt1* expression in embryonal carcinoma (EC) and ES cells (Scharnhorst *et al.*, 1997). It was decided to induce *Wt1* expression in this manner to enable the detection of mutant and wild-type *Wt1* RNA and protein.

4.3.1 Determination of optimum level of retinoic acid needed to induce *Wt1* expression in ES cells.

Before treating the ES cells with retinoic acid to obtain *Wt1* expression, the concentration of retinoic acid that gave the highest level of *Wt1* transcripts with a suitably low level of toxicity needed to be determined. Wild-type ES cells were seeded out into 75cm^2 flasks, and retinoic acid applied with the cell culture medium for four days. Retinoic acid was added in three different concentrations, 1, 5 and

10μM prepared from a stock solution in DMSO. Additional flasks were also set up in parallel, with normal tissue culture medium containing LIF, and normal tissue culture medium without LIF, both with the appropriate concentration of DMSO to control for any associated effects.

Total RNA was extracted from the cells after differentiation for four days. PolyA$^+$ RNA was then purified and separated on an agarose gel. After Northern transfer, the filter was hybridised first with a *Wt1* cDNA probe, then stripped and reprobed with a probe specific to *β actin* RNA, to determine relative levels of loading in each lane (figure 4.12). No signal was seen with the *Wt1* probe in the lane containing RNA from undifferentiated ES cells, and only a very weak signal was seen in the lane from the cells from which LIF had been withdrawn. In the lanes containing RNA from cells to which retinoic acid had been added, the weakest signal relative to the β actin signal was seen in the 1μM lane. There was very little difference seen between the 5 and 10μM lanes.

It was thus demonstrated that all-trans retinoic acid could be used to induce *Wt1* expression, enabling its detection in mutant and wild-type ES cells. From this result, it was decided to use 5μM retinoic acid to induce differentiation of ES cells, as increasing this level to 10μM had no obvious effect on *Wt1* upregulation, and a lower retinoic acid concentration was desirable, to keep any associated toxicity to a minimum.

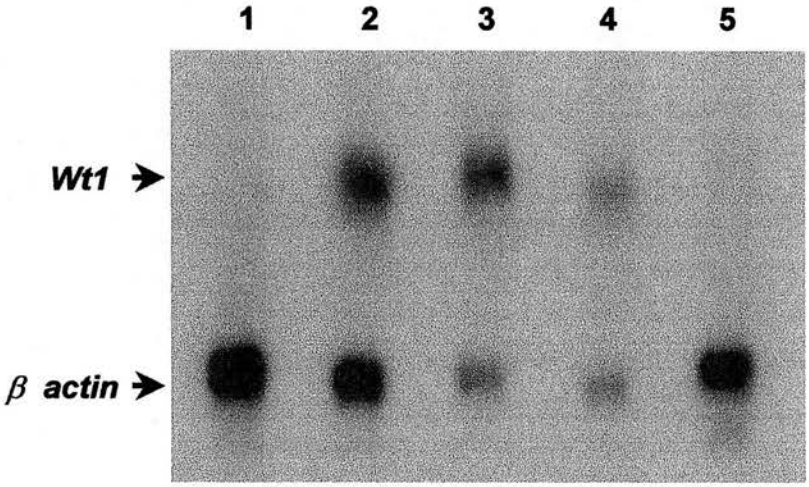


Figure 4.12 Determination of concentration of retinoic acid to use for cell differentiation.

Wild- type CGR8 ES cells were grown for 5 days under the following conditions:

Lane 1: LIF + 0.05% DMSO

Lane 2: 1μM retinoic acid + 0.05% DMSO

Lane 3: 5μM retinoic acid + 0.05% DMSO

Lane 4: 10μM retinoic acid + 0.05% DMSO

Lane 5: 0.05% DMSO

Poly A^+ RNA was extracted from each and separated by electrophoresis on a formaldehyde gel. After transfer to a nylon membrane, the RNA was first hybridised to a cDNA probe specific to *Wt1*. The membrane was then stripped and reprobed with a probe specific to *β actin* to control for unequal lane loading.

4.3.2 Northern analysis of RNA from RA-treated ES cell progeny

After the level of retinoic acid needed for the differentiation of ES cells was established, wild-type, DDS heterozygous and DDS compound heterozygous CGR8 ES cells were induced to differentiate in this way.

Cells of each genotype were seeded out into large (162 cm^2) flasks, then 5μM retinoic acid was added for four days. Control flasks with normal tissue culture medium, containing LIF, were set up in parallel. Total RNA for RNA analysis was then extracted from some of the cells and separated on an agarose gel. After Northern transfer, the resulting filter was hybridised with probes specific to *Wt1* and β actin.

Unfortunately, although many attempts were made to distinguish between mutant and wild-type *Wt1* RNA by Northern analysis, none was successful. This was despite using RNA which was clearly of good quality and which gave a clear signal with a *β actin* probe. Use of poly A^+ RNA instead of total RNA also failed to produce a signal.

4.3.3 Mutant and wild-type WT1 protein detected by Western analysis after treatment of ES cells with retinoic acid

After the conditions required for the detection of *Wt1* expression in ES cells had been determined, cells were differentiated with 5μM RA for the production of protein for Western analysis.

Wild-type, DDS heterozygous and DDS compound heterozygous cells were each seeded out into 6 medium (75cm^2) flasks. These were then grown in the presence of 5μM all-trans retinoic acid for four days before being harvested. Nuclear

protein extracts were produced from the differentiated cells. 75μg of this protein was separated using a 10% SDS-PAGE gel, then transferred on to a nitrocellulose membrane, and the presence of WT1 protein detected using the WT1 N-terminal 6F-H2 antibody (Dako).

Before use of the WT1 antibody to detect protein, the filter was stained using an Instaview Nitrocellulose kit (BDH). This reveals how much protein is actually present on the membrane, and can be used to see how equally the different lanes are loaded (figure 4.13a).

The 6F-H2 N-terminal WT1 antibody was used to detect both mutant and wild-type WT1 protein (figure 4.13b). Wild-type WT1 protein can clearly be seen in both the wild-type and heterozygous lanes. Mutant protein can be seen in both the heterozygous and compound heterozygous lanes.

Four bands (65, 63, 54, 52kDa) are visible when wild-type WT1 protein is separated on an SDS-PAGE gel. These consist of two doublets, one from each of the two translation initiation sites (see section 1.5.1). The two bands making up each doublet consist of the + (65/63kDa) and – 17aa (54/52kDa) isoforms from each initiation site (the presence or absence of the KTS region can not be resolved using these gels). In the DDS heterozygous cells, both the higher molecular weight mutant bands and lower molecular weight wild-type bands lie in the same region on the filter, so it is not possible to distinguish one pair from the other. In wild-type cells and other WT1-expressing cells such as the M15 cell line, the higher molecular weight doublet (65/63kDa) is less strongly expressed than the other pair (54/52kDa). With this in mind, the relative intensities of the lower molecular weight mutant bands (44/42kDa) and higher molecular weight wild-type bands (65/63kDa) can be

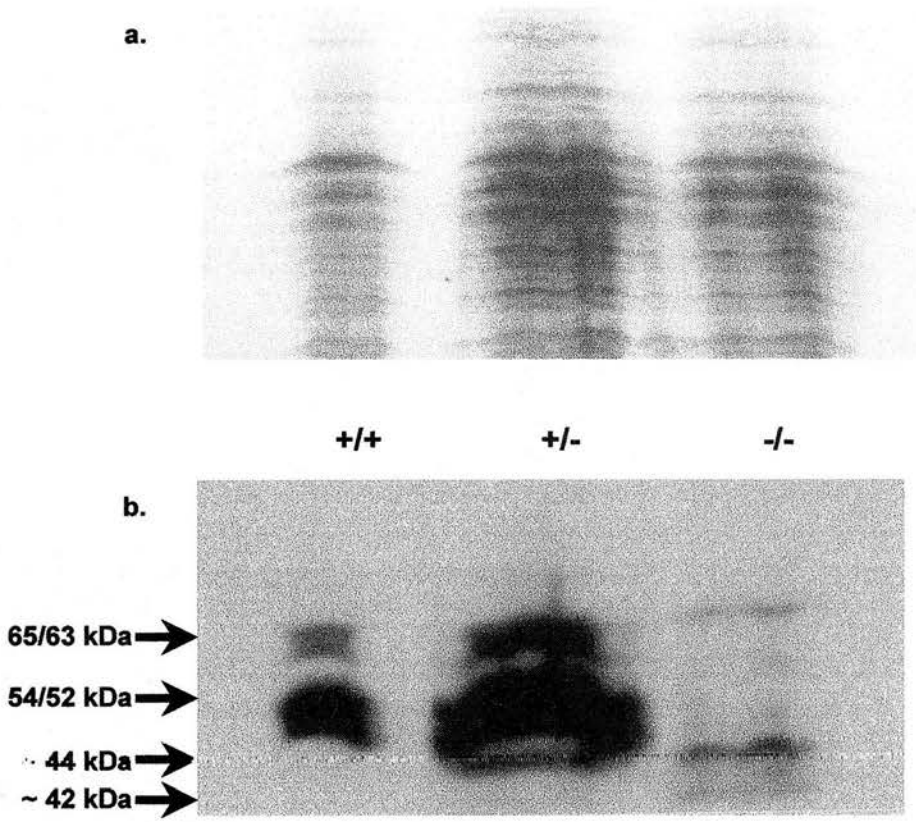


Figure 4.13 Western analysis of nuclear extracts from differentiated ES cells

Wild-type, DDS heterozygous and DDS compound heterozygous CGR8 ES cells were differentiated for 4 days with 5μM all-trans retinoic acid. Nuclear extracts were prepared from the differentiated cells and separated on a 10% SDS-PAGE gel.

a. After Western blotting, the nitrocellulose filter was stained using Instaview (BDH) to determine how equally loaded the lanes were.

b. WT1 protein was then detected using the 6F-H2 antibody.

compared in heterozygous cells. This reveals a huge excess of wild-type over mutant protein.

Comparing the relative levels of protein loading using the stained filter (figure 4.13a), it can be seen that protein from the three genotypes is present on the filter at approximately equal levels. This means that the observed scarcity of WT1 protein in the DDS compound heterozygous lane indicates a genuine reduction in the amount of mutant protein in the cells. It can be seen from figure 4.13 that the level of lower molecular weight WT1 protein (44/42kDa) in the compound heterozygous cells approximates to that in the heterozygous cells. As this represents only a tiny fraction of the total amount of WT1 protein in the heterozygous lane, but the majority of that in the compound heterozygous lane (in which it is being produced from two alleles, rather than one), it can be concluded that, although DDS compound heterozygous cells do contain WT1 protein, it is found at a much lower level than the WT1 protein in the wild-type and DDS heterozygous cells.

A significantly lower level of mutant than wild-type protein had previously been shown to be present in DDS heterozygous cells (Patek *et al.*, 1999). This occurred despite equivalent levels of mutant and wild-type RNA. When heterozygous ES cells were made homozygous using high G418 selection, no WT1 protein was detected, despite the presence of mutant mRNA being demonstrated by Northern analysis (C.E. Patek, personal communication). The scarcity of mutant protein in the nuclear extracts is shown here by Western analysis not to be due to abnormal localisation to the cytoplasmic fraction (figure 4.14).

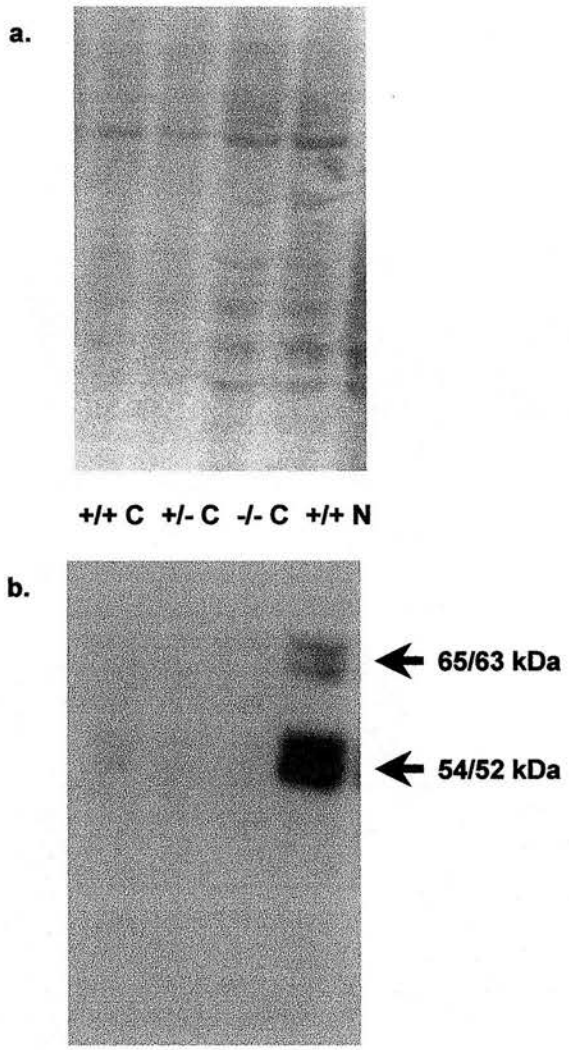


Figure 4.14 Western analysis of cytoplasmic extracts from differentiated ES cells.

To demonstrate that the lack of WT1 mutant nuclear protein in DDS heterozygous and compound heterozygous ES cells is not due to its sequestration in the cytoplasm, Western analysis with the 6F-H2 WT1 antibody was performed on cytoplasmic extracts from these cells. CGR8 ES cells were differentiated as before, then separated on a 10% SDS-PAGE gel.

a. After blotting, the filter was stained with Instaview to determine levels of loading.

b. Analysis with the 6F-H2 antibody. Nuclear protein from RA-differentiated wild-type ES cells was included as a positive control.

If the situation in the compound heterozygous cells resembles that in the high G418 homozygous cells, with mutant mRNA found at appropriate levels and yet a lower than expected amount of mutant protein detected, some kind of post-translational mechanism is at work, possibly affecting mutant protein stability. The relatively high levels of mutant protein found in heterozygous cells, as compared with homozygous cells, may indicate that the presence of WT1 wild-type protein in the same cell stabilises the DDS mutant form in some way.

4.3.4 Immunofluorescence analysis of ES cells of different DDS genotypes after differentiation with retinoic acid

Having established that treatment with all-trans retinoic acid causes induction of *Wt1* expression in ES cells, it was decided to investigate further the actual effect of the RA on the cells. The mode of action of RA on *Wt1* expression in ES cells is, as yet, unknown, but is believed not to be a direct action on *Wt1* transcription via RA-receptors (Scharnhorst *et al.*, 1997). Immunofluorescence analysis was used to enable the *Wt1* expression status of individual cells to be determined. It could then be seen whether *Wt1* expression in retinoic acid-treated cells is universal, or only a property of a subset of the cells, those which have differentiated along a particular developmental pathway. The morphology of specific cells and whether or not they were expressing WT1 could be observed and related.

In an initial experiment, performed to determine whether immunofluorescence analysis could detect WT1 protein in differentiated ES cells, wild-type ES cells only were grown on glass cover-slips both in the presence and in the absence of retinoic acid. After four days under these conditions, the cells were

washed, fixed and blocked, then the C-terminal C-19 WT1 primary antibody applied. This antibody was used because it was known to give the strongest signal in immunofluorescence analysis. The secondary antibody was FITC-conjugated, enabling the location of the WT1 protein to be detected using a fluorescence microscope.

When undifferentiated ES cells were examined, the level of FITC observed approximated background level, supporting the previous observation that undifferentiated ES cells do not express WT1 (figure 4.15d). When differentiated wild-type cells were examined, virtually all cells were seen to contain WT1 protein at high levels in their nuclei (figure 4.15b).

As a further comparison of relative levels of WT1 in cells of different DDS genotypes, cells of all three genotypes were then differentiated with retinoic acid on coverslips for 4 days. The cells were then washed, fixed and blocked, then the N-terminal WT1 antibody 6F-H2 was applied. This detects both mutant and wild-type forms of WT1. A FITC-conjugated secondary antibody was used, causing the location of WT1 protein to be marked by green fluorescence. The mountant used when the coverslips were mounted on to glass slides contained DAPI, enabling cell nuclei to be identified.

Examination of RA-differentiated wild-type and DDS heterozygous cells revealed that *Wt1* expression occurs in virtually all cells (figure 4.16a-d). Expression was far less in compound heterozygous cells, with the vast majority of cells again expressing *Wt1*, but at far lower levels (figure 4.16e & f). Signal intensities are comparable, as images were captured using the same exposure times and settings. This agrees with the data obtained by Western analysis, which showed far lower

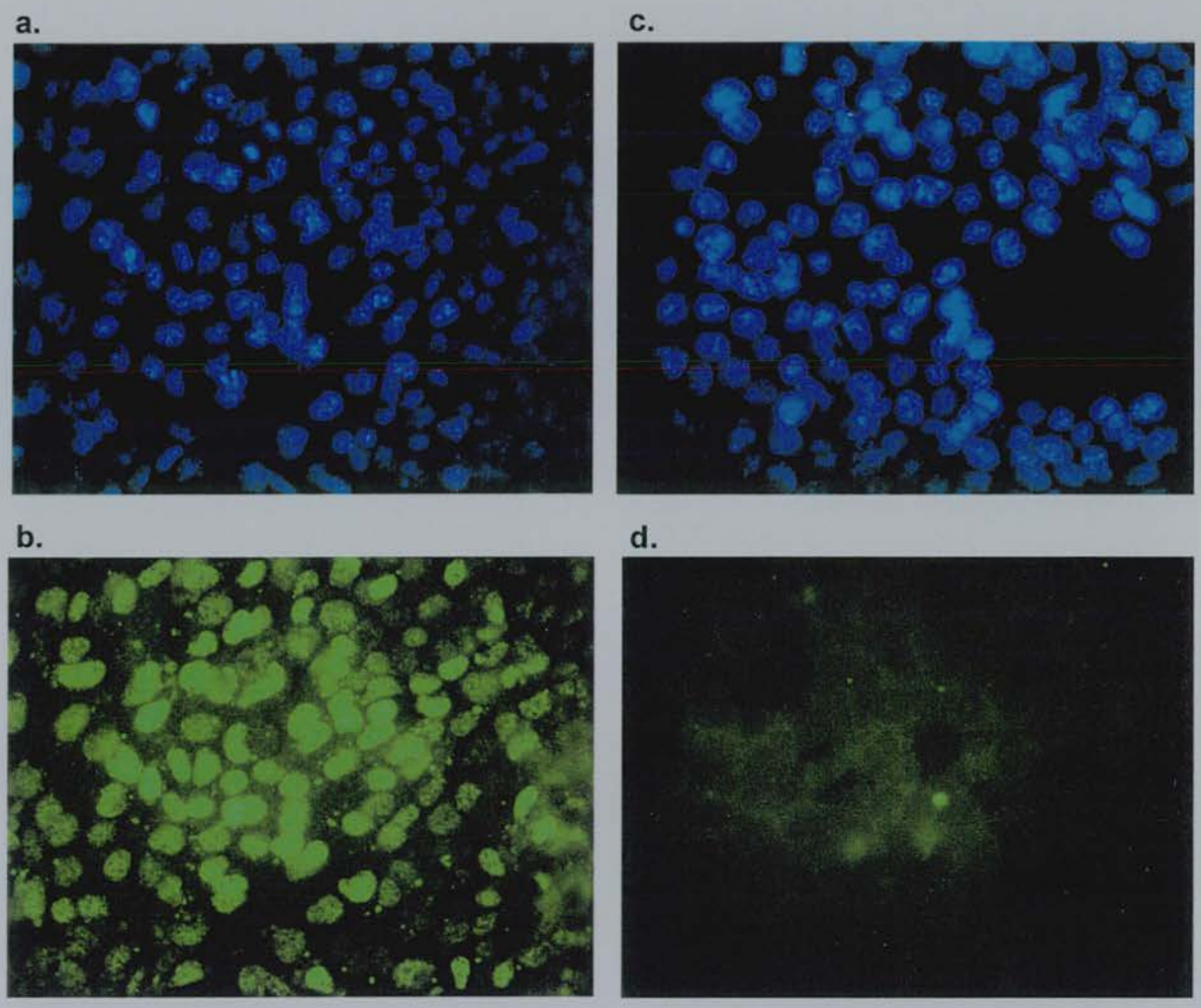


Figure 4.15 Immunofluorescence analysis of WT1 expression in differentiated and undifferentiated wild-type CGR8 ES cells.

a. RA-differentiated cells, DAPI staining

b. RA-differentiated cells, C19 WT1 primary antibody, FITC-conjugated secondary antibody

c. Undifferentiated ES cells, DAPI staining

d. Undifferentiated ES cells, C19 WT1 primary antibody, FITC-conjugated secondary antibody

Magnification x400.

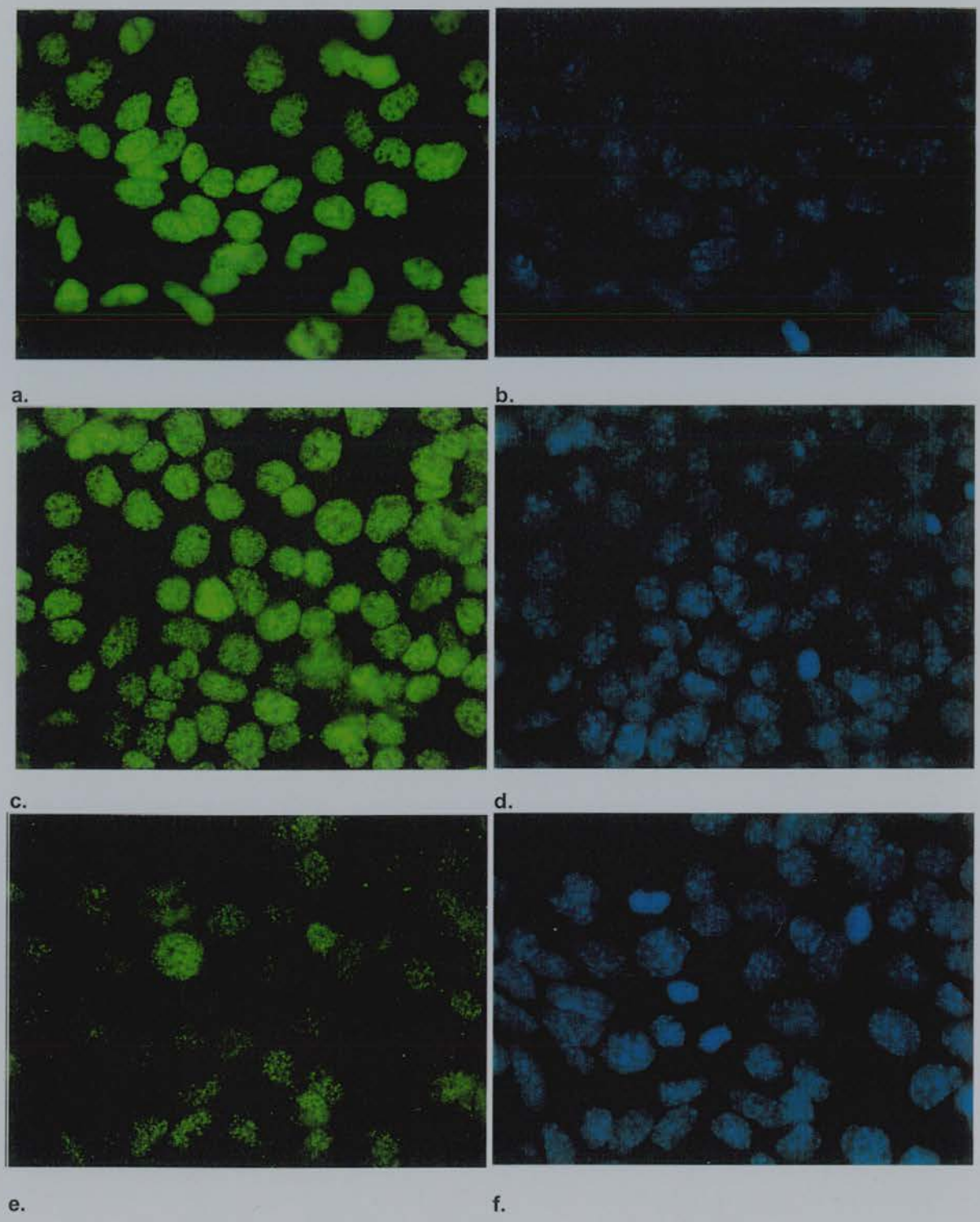


Figure 4.16 Examination of WT1 protein in RA-differentiated ES cells of different DDS genotypes using immunofluorescence analysis

ES cells were differentiated on glass cover slips for 4 days with 5μM RA. Immunofluorescence analysis was then performed using the 6F-H2 WT1 primary antibody and a FITC-conjugated secondary antibody. Magnification x400.

a. Wild-type cells, 6F-H2
b. Wild-type cells, DAPI
c. Heterozygous cells, 6F-H2
d. Heterozygous cells, DAPI
e. Compound heterozygous cells, 6F-H2
f. Compound heterozygous cells, DAPI

WT1 protein levels in the sequentially targeted cells than would be expected from the amount of protein loaded. The immunofluorescence evidence reveals that this low level is due to universal lowered expression, rather than a small proportion of cells expressing *Wt1* at levels comparable to the other two genotypes.

The presence of WT1 signal in nearly all differentiated cells may indicate that retinoic acid acts directly on *Wt1* transcription, or at least upstream of something acting on *Wt1* transcription, rather than causing the cells to differentiate into different types, some of which normally express *Wt1*. To examine this more closely, experiments were designed to examine *Wt1* expression immediately after addition of retinoic acid, as well as in retinoic acid-differentiated cells after retinoic acid had been withdrawn.

4.3.5 Immunofluorescence analysis of cells after addition of retinoic acid reveals when WT1 protein appears.

An idea of the mechanism by which RA causes *Wt1* induction in differentiating ES cells could be obtained by determining the length of time taken after RA addition for WT1 protein to be detected. A rapid reaction of *Wt1* to RA would suggest a direct action of RA on *Wt1* transcription, whereas a delay would imply that the observed *Wt1* expression results from the differentiation status of the cells.

To determine how soon after the addition of retinoic acid to undifferentiated ES cells WT1 expression begins, wild-type CGR8 ES cells were differentiated on glass cover slips with retinoic acid for lengths of time ranging from 12 hours to 4

days. Immunofluorescence analysis was then performed using the WT1 C19 antibody to detect the appearance of WT1 protein.

WT1 protein was first seen in a few cells after 36 hours of exposure to retinoic acid (figure 4.17). By 48 hours of exposure, the vast majority of the cells were seen to express WT1. It was not possible to determine whether individual cells seen to be expressing WT1 had a more differentiated morphology than their non-expressing neighbours using phase contrast microscopy, but by the time WT1 expression was universal, it was clear that the cells had generally lost their initial ES cell-like appearance (figure 4.18). However, the length of time taken before WT1 expression appeared suggests that the activation of *Wt1* is not a direct result of retinoic acid addition, but caused by differentiation of the cells.

4.3.6 Immunofluorescence analysis of retinoic acid-differentiated cells after retinoic acid withdrawal.

Addition of all-trans retinoic acid to ES cells causes induction of WT1 expression, which first appears in some cells after 36 hours of exposure, then quickly spreads to all cells by 48 hours. The seeming unrelatedness of this expression to cell morphology (i.e. cells of very different appearance express *Wt1*) suggests that retinoic acid-induced differentiation into particular cell types that normally express *Wt1* is not the mechanism in action here. However, the slow response of *Wt1* expression to RA indicates that RA probably does not directly affect *Wt1* transcription. In an attempt to clarify this, the effects of differentiation with retinoic acid followed by its removal were examined. This would indicate whether the continued presence of retinoic acid in the cell culture medium is necessary for *Wt1*

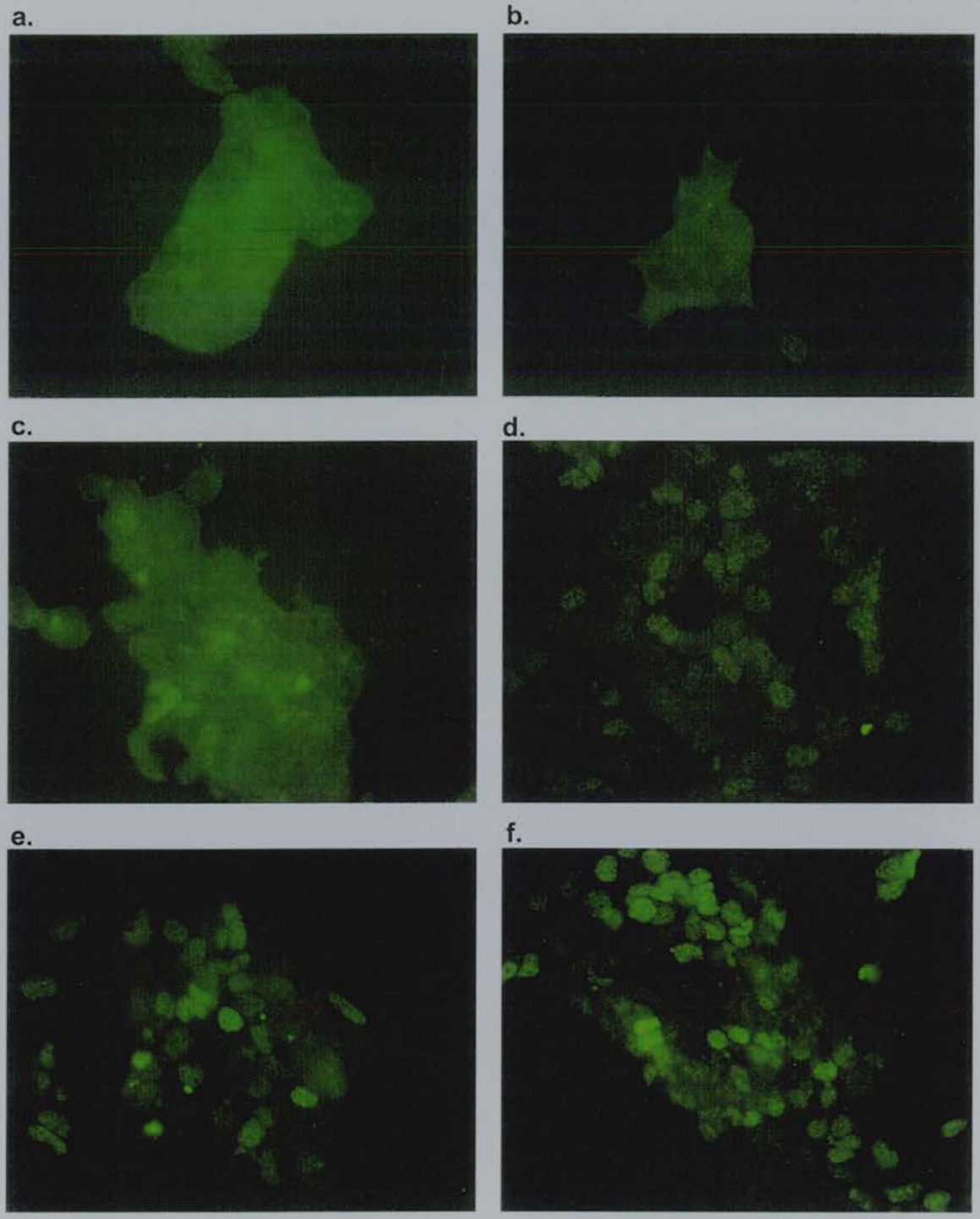


Figure 4.17 Induction of WT1 expression by the addition of retinoic acid to wild-type ES cells

Using C19 WT1 antibody. Magnification x400. Time after retinoic acid addition:

a. 12 hours b. 24 hours c. 36 hours

d. 48 hours e. 72 hours f. 96 hours

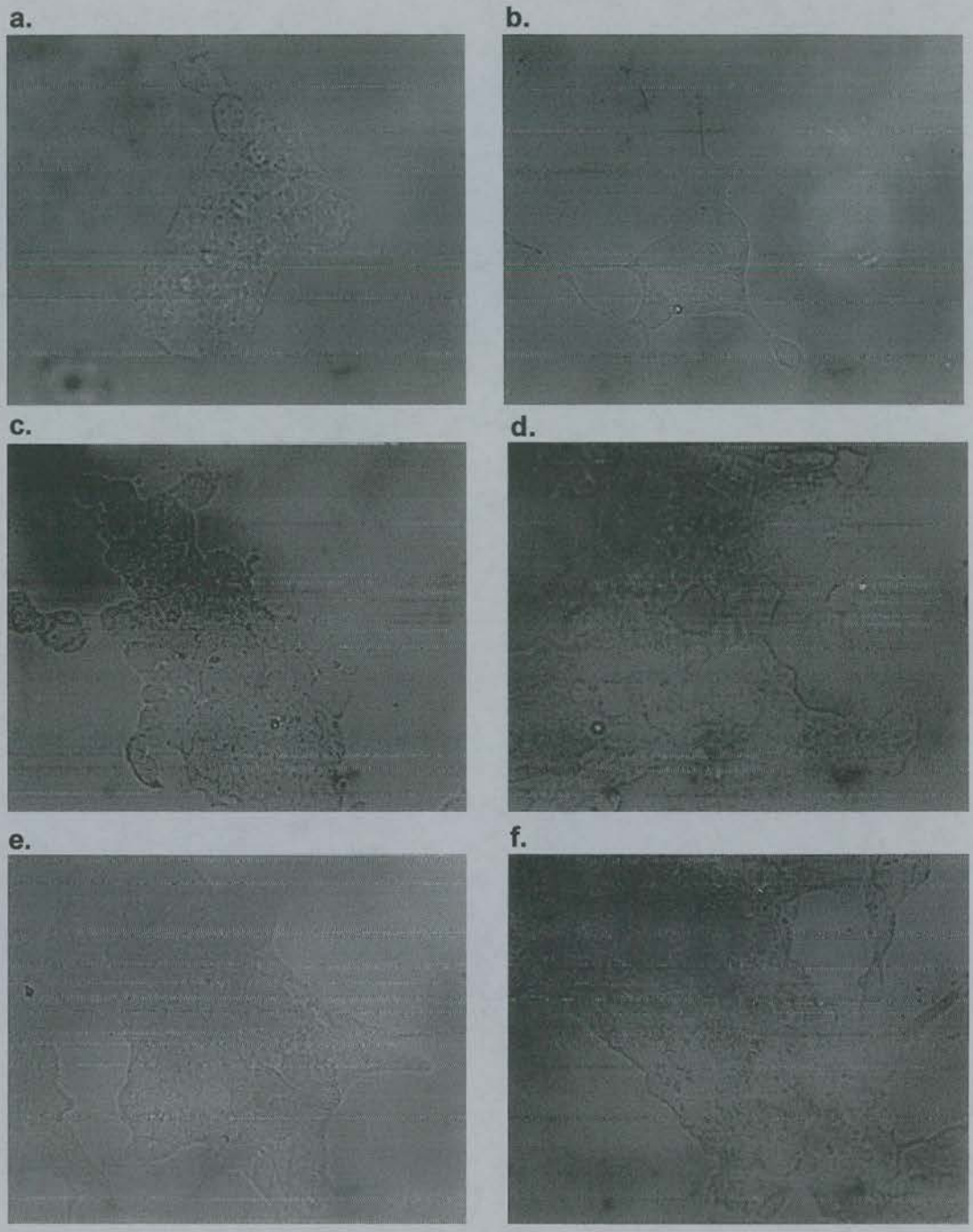


Figure 4.18 Cell morphology after addition of retinoic acid to wild-type ES cells

Time after retinoic acid addition:

a. 12 hours **b**. 24 hours **c**. 36 hours

d. 48 hours **e**. 72 hours **f**. 96 hours

Magnification x400.

expression, thus showing that the effect is a direct one, or if *Wt1* expression continues, pointing to the differentiated nature of the cells as the cause.

Wild-type ES cells were differentiated with retinoic acid for 4 days. The medium was then replaced with normal tissue culture medium, containing LIF, for lengths of time ranging from zero to 10 days. Immunofluorescence analysis was then performed using the C19 WT1 antibody. WT1 protein was found in the vast majority of cells up to 10 days after the withdrawal of retinoic acid (figure 4.19). This indicates that after the cells have been differentiated for 4 days with retinoic acid, the continued presence of retinoic acid is no longer necessary for the cells to maintain *Wt1* expression.

This suggests that the action of retinoic acid on *Wt1* expression does not occur directly on *Wt1* transcription, unless some type of positive feedback loop is triggered which continues to function once the RA is removed. A more likely mode of action is indirectly through differentiation of the ES cells. This suggests that the presence of RA in the cell culture medium causes the cells to differentiate into cell types of different morphologies, which nonetheless all express *Wt1*. Whether or not the cells continue to differentiate after the removal of the RA, the *Wt1* expression remains constant for at least 10 days after this point. This observation is unexpected, considering that very few cell types express *Wt1* during development.

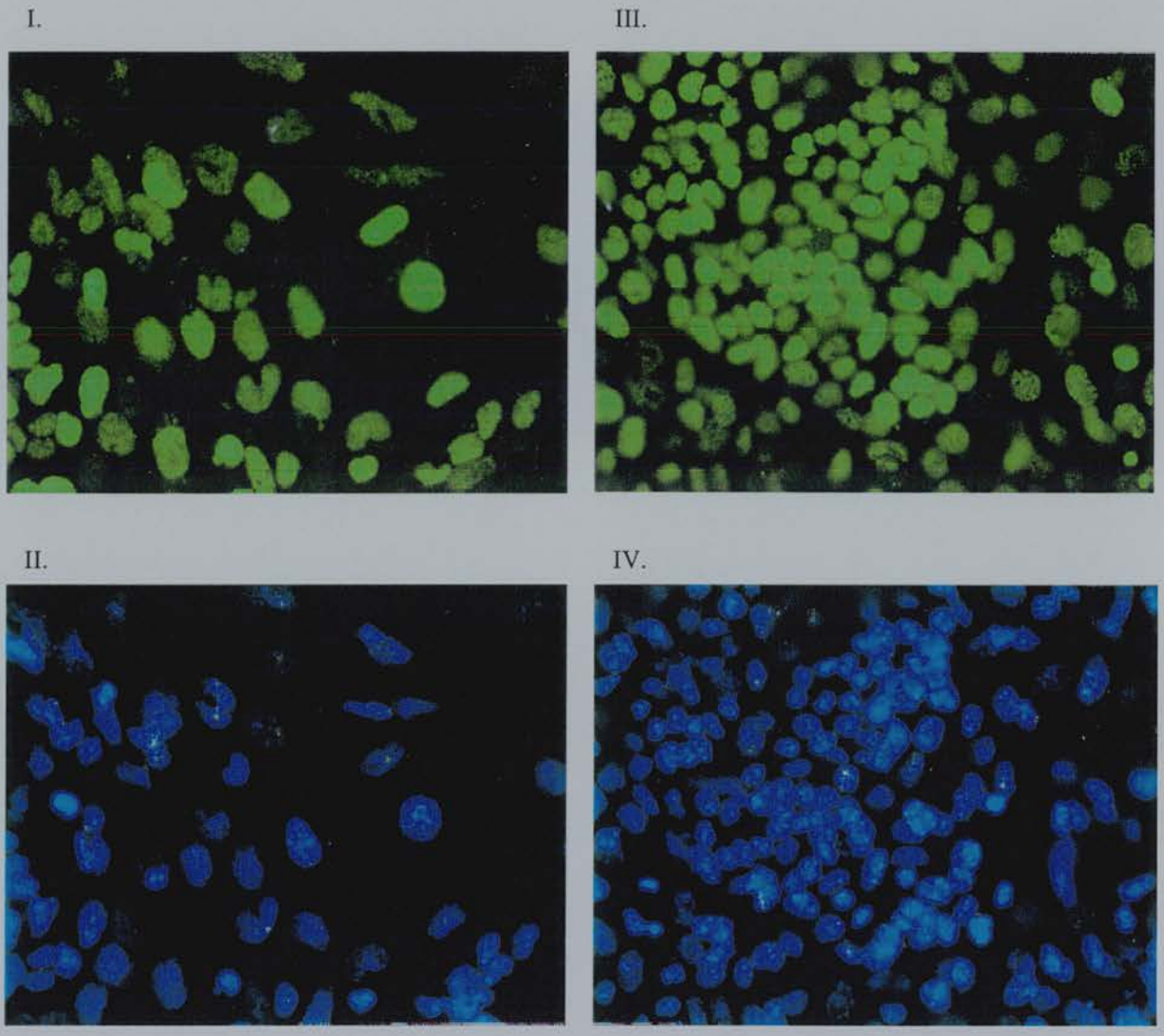


Figure 4.19a Effects of withdrawing retinoic acid from differentiated ES cells for 0 and 2 days

I. 0 days, C19 WT1 antibody **III**. 2 days, C19 WT1 antibody

II. 0 days, DAPI **IV**. 2 days, DAPI

Magnification x400.

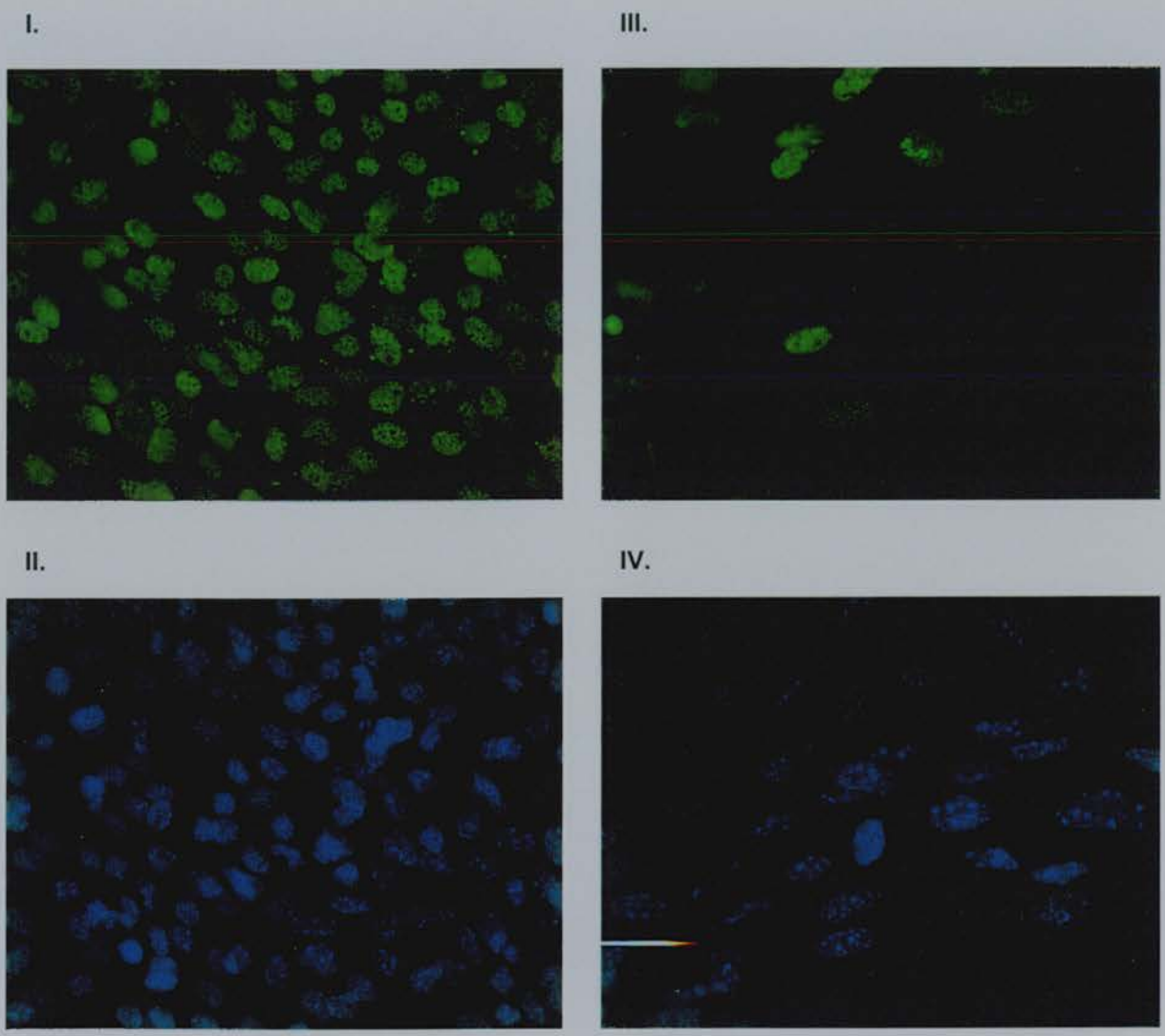


Figure 4.19b Effects of withdrawing retinoic acid from differentiated ES cells for 4 and 6 days

Magnification x400.

I. 4 days, C19 WT1 antibody **III**. 6 days, C19 WT1 antibody

II. 4 days, DAPI **IV**. 6 days, DAPI

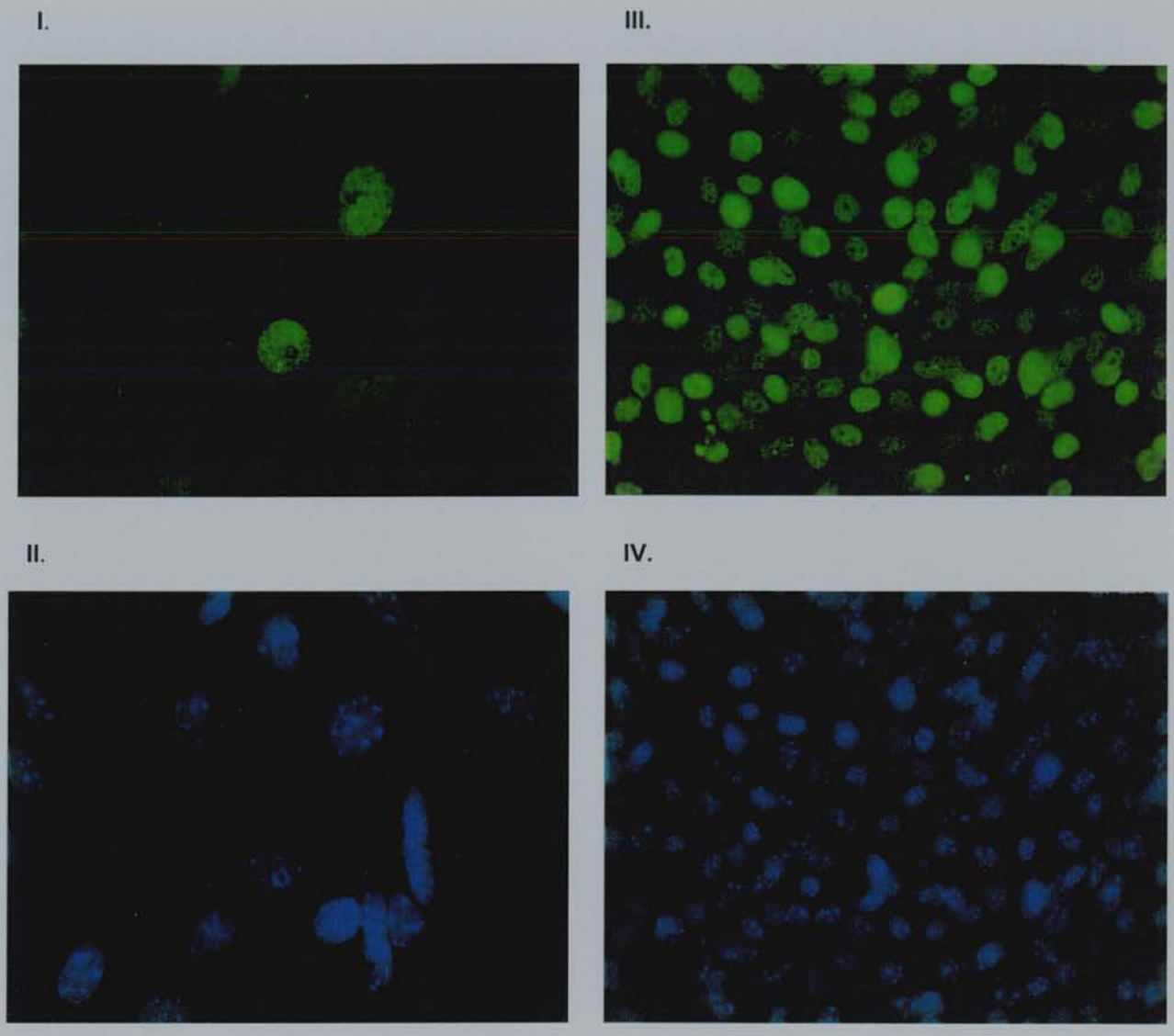


Figure 4.19c Effects of withdrawing retinoic acid from differentiated ES cells for 8 and 10 days

Magnification x400.

I. 8 days, C19 WT1 antibody **III**. 10 days, C19 WT1 antibody

II. 8 days, DAPI **IV**. 10 days, DAPI

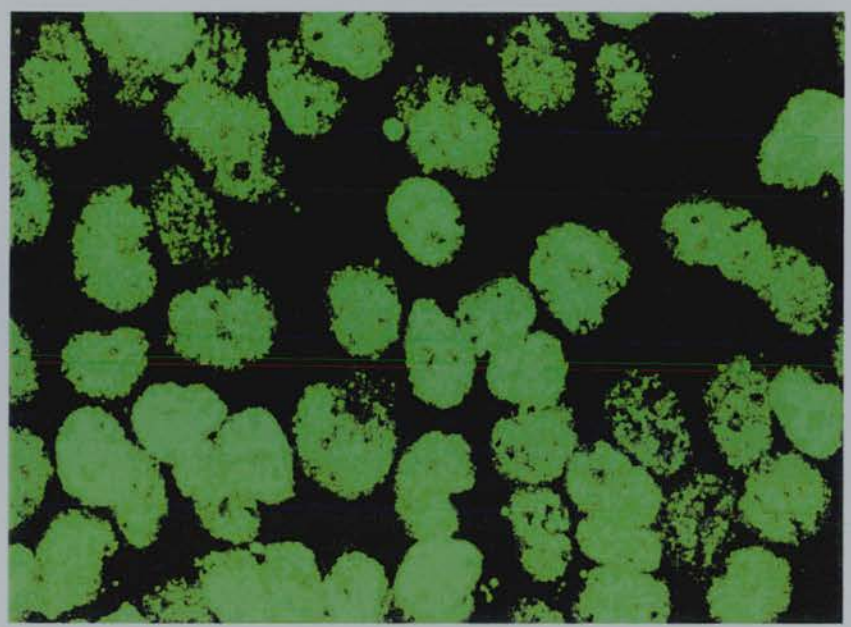
4.4 Effects of DDS mutations on subnuclear localisation and ratios of Wt1 splice isoforms

4.4.1 Examination of the subnuclear localisation of WT1 protein by immunofluorescence analysis in differentiated CGR8 ES cells

Denys-Drash syndrome-like mutant proteins have been shown to colocalise with +KTS isoforms and splicing factors (Larsson *et al.*, 1995). As these mutant proteins have been suggested to act by binding to wild-type WT1 protein present in the same cell and disrupting its normal function, it was hypothesised that this binding leads to the colocalisation with splicing factors of all WT1 protein in a DDS cell. Indeed, Englert *et al.* (1995) found that coexpression of wild-type and truncated WT1 protein in a cell resulted in an alteration of wild-type protein distribution to a 'speckled' pattern. This would prevent the wild-type WT1 present from performing its normal functions as a transcription factor, but splicing functions might be left intact.

To investigate whether this effect of truncated WT1 protein on wild-type protein occurs in DDS heterozygous cells, wild-type and DDS heterozygous CGR8 ES cells were grown on glass cover slips in six-well plates for four days in the presence of retinoic acid. After fixation and blocking, the C-19 C-terminal WT1 antibody was used to detect the subnuclear locations of the wild-type protein only, and the 6F-H2 N-terminal antibody to detect both mutant and wild-type WT1 proteins (figures 4.20 & 4.21).

a.



b.

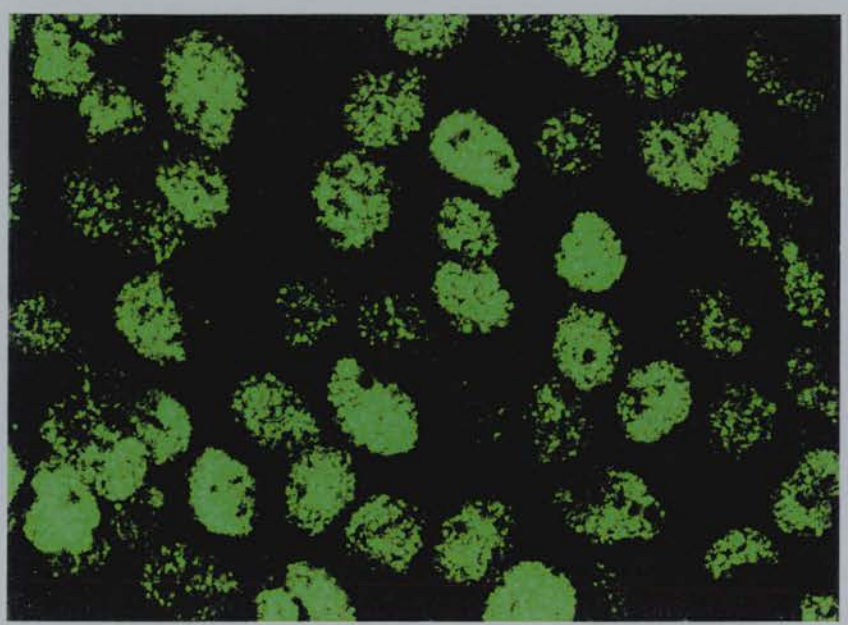


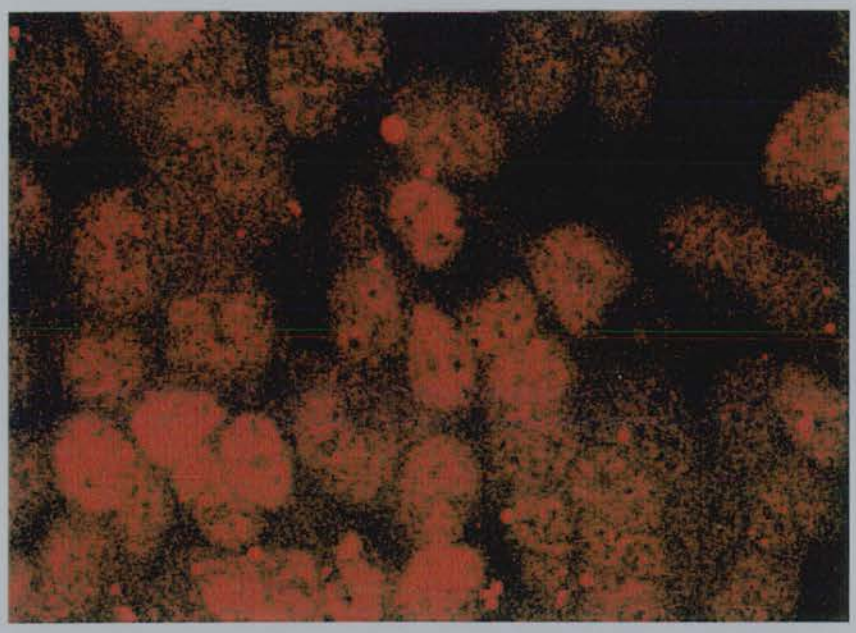
Figure 4.20 Immunofluorescence analysis of RA-differentiated CGR8 wild-type and DDS heterozygous ES cells using the 6F-H2 WT1 antibody.

Cells were grown and differentiated on glass cover slips, then immunofluorescence analysis was carried out using the 6F-H2 WT1 antibody with a FITC-conjugated secondary antibody. This antibody binds to the N-terminal region of WT1, so will detect both mutant and wild-type WT1 protein in the DDS heterozygous cells. Examination of the subnuclear localisation of the protein was carried out using a confocal microscope.

a. Wild-type cells.
b. DDS heterozygous cells.

Magnification x400.

a.



b.

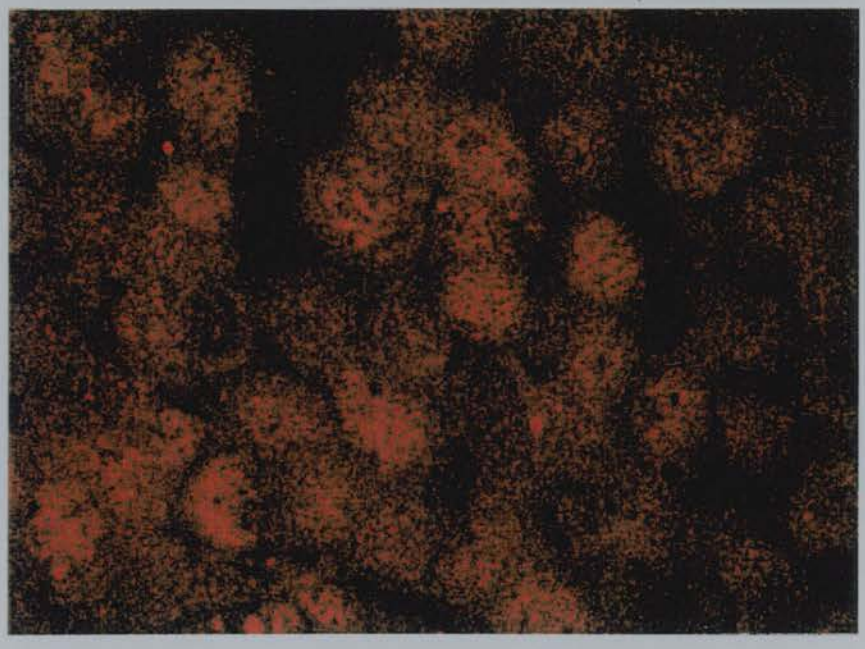


Figure 4.21 Immunofluorescence analysis of RA-differentiated CGR8 wild-type and DDS heterozygous ES cells using the C19 WT1 antibody.

Cells were grown and differentiated as in figure 3.20, then immunofluorescence analysis was carried out using the C19 WT1 antibody with a Texas Red-conjugated secondary antibody. This will only detect wild-type WT1 protein in the DDS heterozygous cells, as the antibody will not bind to the truncated form of the protein. The subnuclear localisation of the protein was examined using confocal microscopy. Magnification x400.

a. Wild-type cells

b. DDS heterozygous cells

Examination of the slides using confocal microscopy did not reveal any apparent difference in the subnuclear localisation of the wild-type WT1 protein in the DDS heterozygote. A more speckled appearance was not apparent with either the C-terminal or N-terminal antibody in heterozygous as compared with wild-type cells. Thus, in these cells, endogenous DDS mutant proteins do not appear to affect WT1 function by altering the subnuclear localisation of the endogenous wild-type protein present.

4.4.2 Comparison of ratios of *Wt1* isoforms possessing and lacking first alternatively-spliced region

Studies of the expressed ratios of the different *Wt1* isoforms possessing and lacking the first alternatively spliced region (exon 5) have found species-specific, tissue-specific and developmental stage-specific differences in these ratios (Haber *et al.*, 1991; Renshaw *et al.*, 1997). These studies did not examine ES cells, however, so it was considered interesting to examine these ratios and compare them with those previously reported for mouse tissues (Renshaw *et al.*, 1997). It was already known that differentiated ES cell progeny express WT1 isoforms both with and without alternatively-spliced exon 5, as the 17 amino acid difference in size between these two isoform groups can be detected on a Western blot (figure 4.14b). The proportions of the two isoform groups had not been determined, however, and as the ratios of mutant to wild-type forms changes from RNA to protein in the DDS heterozygote (Patek *et al.*, 1999), and the DDS compound heterozygote expresses very little mutant protein, it was decided to examine the isoform ratios at the RNA level by RT-PCR.

The PCR primer pair K447 and K448 was used in this analysis (Moore *et al.*, 1999)(appendix II). This amplifies a stretch of the *Wt1* cDNA surrounding the exon 5 region, and gives a band of 243bp with isoforms containing exon 5 and 192bp from isoforms without. The PCR reactions were performed with the addition of ^{33}P α-dATP, products were run on a 6% polyacrylamide sequencing gel (see appendix I), then dried and exposed to X-ray photographic film. Relative band intensities were compared using a Gel Doc 1000 gel viewer and Molecular Analyst software (BioRad).

Surprisingly, it was discovered by this method that undifferentiated ES cells did in fact express *Wt1*, but at very low levels relative to retinoic acid-differentiated cells (figure 4.22). This can be accounted for by the higher sensitivity of the RT-PCR technique compared with Western analysis. An exception to this is the cDNA from undifferentiated wild-type E14 ES cells. In this, and subsequent PCRs performed on the same sample, it produced a strong signal, suggesting that either the cDNA concentration was much higher than the other samples, or that the culture contained a proportion of spontaneously differentiated cells, and was therefore expressing higher than expected levels of *Wt1*.

Table 4.4 Ratios of *Wt1* isoforms with and without first alternatively-spliced region shown by RT-PCR analysis of embryoid bodies and RA-differentiated ES cell progeny

Genotype	Isoform ratios - exon 5: + exon 5			
	CGR8 cells	E14 cells	CGR8 EBs	E14 EBs
+/+	0.65	0.36	0.50	0.43
+/-	0.57	0.68	0.56	0.63
-/-	0.65	0.42	0.56	0.61

It was found that all genotypes of RA-differentiated CGR8 cells, plus wild-type, DDS heterozygous and DDS high G418 homozygous E14 cells and embryoid bodies (EBs) produced from those cells (see section 5.2.4) expressed both isoforms. The larger isoforms were more highly expressed, with ratios of the smaller to larger isoforms having a mean value of 0.55 (ranging from 0.36-0.68:1). This is extremely consistent with the median values of 0.28-0.64:1 published by Renshaw *et al.* (1997) for various mouse tissues. No difference in this ratio was seen that could be described as attributable to *Wt1* genotype.

4.4.3 Comparison of ratios of Wt1 isoforms possessing and lacking the KTS alternatively-spliced region

The relative ratios of + and - KTS isoforms of WT1 in embryonic stem cells has not previously been examined. These two groups of isoforms, being only three amino acids different in size, are not readily separable as proteins on an acrylamide gel, so it was previously undetermined whether or not both isoforms were expressed

at all in differentiated ES cells. Previous studies had found both these groups of isoforms expressed in all tissues examined, including Wilms' tumours, and human foetal kidney and mouse adult ovary, testis and uterus (Haber *et al.*, 1991; Renshaw *et al.*, 1997). Haber *et al.* (1991) found that isoforms containing the KTS insert were always seen to be in the majority, in an average ratio of 3.3:1 with the isoforms lacking KTS. This ratio is somewhat higher than those found in subsequent studies (Simms *et al.*, 1995; Renshaw *et al.*, 1997).

Again, this analysis was carried out by RT-PCR, as described above, with ^{33}P-labelled products being separated on a 6% polyacrylamide denaturing gel. The primers used were J520 and J521, as described in Moore *et al.* (1999), which bind within exon 9. For sequences and PCR conditions, see appendix II.

As the KTS region falls after the *Rsr*II site into which the selectable marker sequences are inserted in the WTV1 and WTV2 targeting vectors, it was expected that no bands would be produced from the compound heterozygous cDNA (figure 4.23). The inserted region in each vector is approximately 2kb, and contains a polyadenylation signal, so transcriptional read-through would not be expected. Heterozygous clones would also only be expected to produce signal from one *Wt1* allele. This was not the case, however, and cDNA from cells of all genotypes (including E14 heterozygous and high G418 homozygous cells) was found to produce the expected doublet of 115 and 106bp bands (figure 4.24). To confirm that this result was due to transcriptional read-through, and not to use of the incorrect genotype of cells, a second pair of primers was designed in this region. This primer pair, DDS1 and DDS2, spanned the *Rsr*II site, so a signal would not be obtained from alleles which contained the insert, as the primers would be too far apart (see

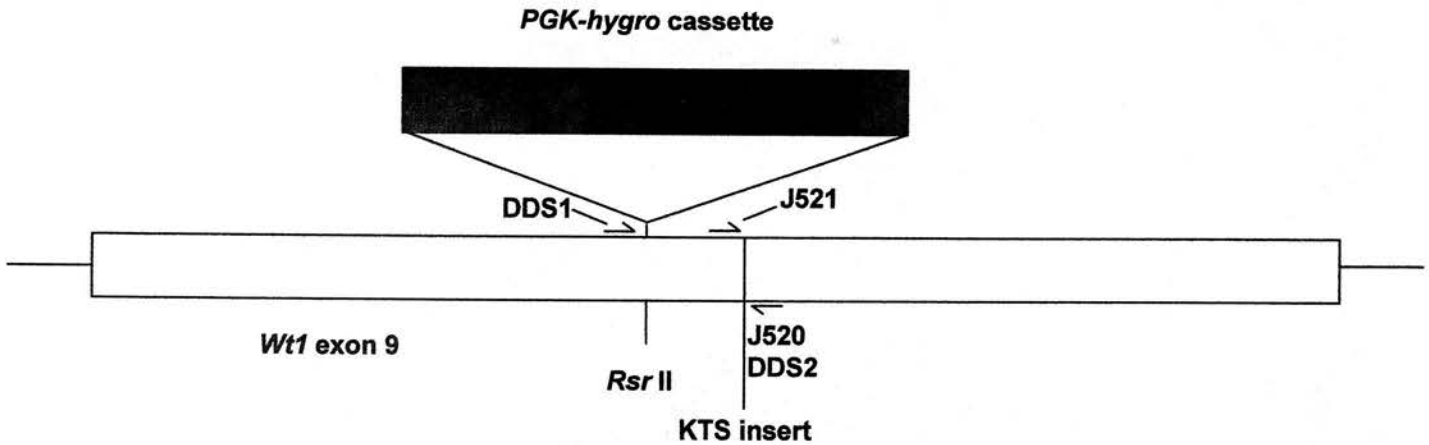


Figure 4.23 Exon nine of *Wt1*, showing positions of primers used in RT-PCR analysis of +/-KTS isoforms

The figure shows the position of the *Rsr*II site in exon 9 of *Wt1*, into which the *PGK-hygro* cassette of vector WTV2 is inserted (not to scale). The positions of primer pair J520 and J521 (Moore *et al.*, 1999) are indicated, as are primer pair DDS1 and DDS2, which span the *Rsr*II site, and therefore give no product from targeted alleles.

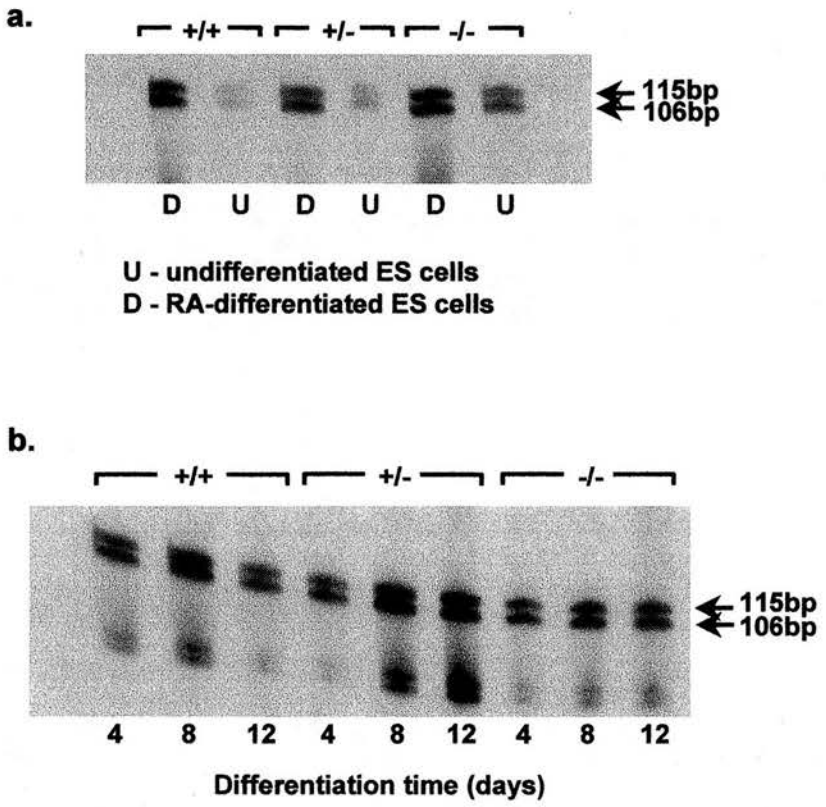


Figure 4.24 RT-PCR analysis of CGR8 RA-differentiated ES cells and embryoid bodies using primers around the +/- KTS alternative splice site

Autoradiographs from PCRs performed with primers J520 and J521 (Moore *et al*, 1999).

a. CGR8 undifferentiated and RA-differentiated ES cells

b. CGR8 embryoid bodies

+/+ wild-type

+/- DDS heterozygote

-/- DDS compound heterozygote

appendix II for primer sequences and PCR conditions). With this pair of primers, the expected result was obtained: a pair of bands of expected sizes 164 and 173bp were produced from wild-type and heterozygous, but not compound heterozygous or homozygous cDNA (fig 4.25). This was only examined by separation on an ethidium bromide stained gel, as it was merely the presence or absence of the bands which was sought.

The bands produced by the +/- KTS primers (J520 and J521) from the compound heterozygous and homozygous DDS cDNA (and presumably from the cDNA from one allele of the heterozygous cells) appeared to originate from a transcript under the control of the *Wt1* promoter, as the bands were far stronger in the differentiated than undifferentiated cDNA. Unfortunately, as the precise nature of the RNA transcript which is being amplified here is unknown, the relative proportions of the + and – KTS transcripts derived from the wild-type allele in the heterozygous cells cannot be reliably determined using primers J520 and J521. This could have been done using primers DDS1 and DDS2, but time did not permit. Therefore only the +/- KTS ratio in the wild-type cells can be examined. These results, for RA-differentiated ES cells and embryoid bodies (see section 5.2.4) are presented in table 4.5.

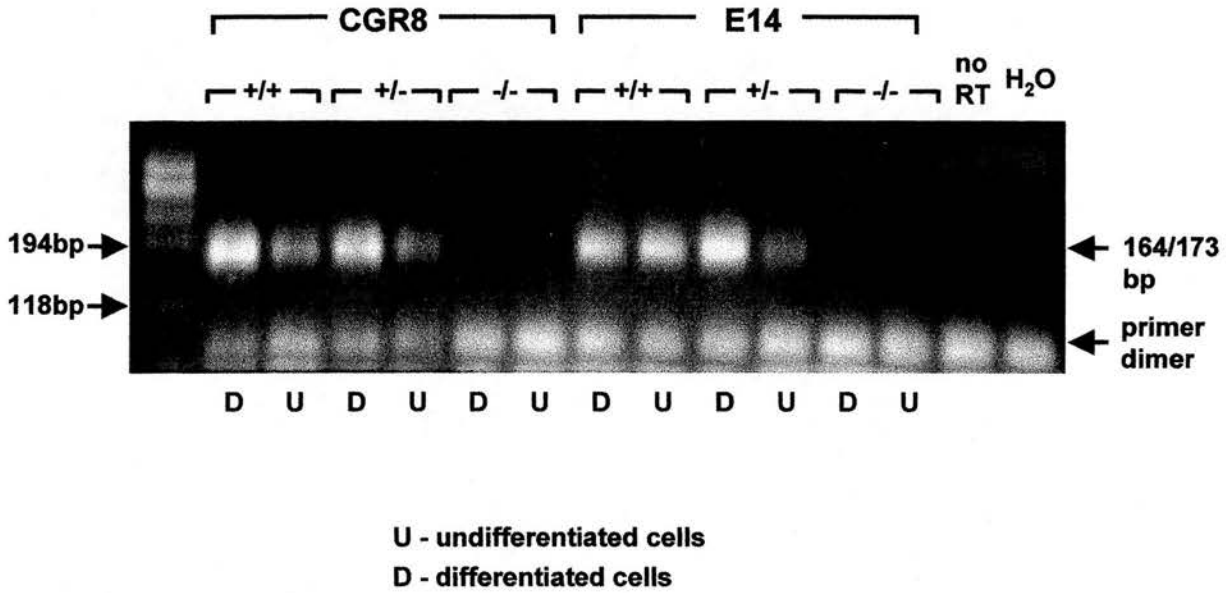


Figure 4.25 RT-PCR to confirm genotype of sequentially targeted cells

As unexpected positive signals were produced by cDNA from DDS compound heterozygous and homozygous cells, a PCR was designed which crossed the site at which the selectable marker sequences were inserted in WTV1 and WTV2. This should not amplify sequences from targeted alleles, as the insertion places 2kb of sequence between the two primers. Results are shown for differentiated and undifferentiated cells from both the E14 and CGR8 cell lines, revealing that no bands were seen in either the compound heterozygous or homozygous cDNA.

Table 4.5 Ratios of KTS isoforms in wild-type embryoid bodies and RA-differentiated ES cells

Cell line	Ratio +KTS:-KTS isoforms	
	Cells	Embryoid bodies
CGR8	0.71	0.91
E14	n/d	0.83

n/d – not determined

Renshaw *et al.* (1997) found no consistent ratio differences between species, tissue or developmental stage for these isoforms, with median values for the mouse ranging from 1.04-1.41. The ratios in table 4.5 are also fairly similar to one another, although these are lower than those found by Renshaw *et al.*, with a higher proportion of transcripts without the KTS insert found.

4.5 Discussion of chapter 4

The results described above indicate that the cells used for sequential targeting began the experiment with the correct genotype and a high proportion of cells containing the correct number of chromosomes. The identity of the plasmid, WTV2, used to produce the second targeting event was also confirmed before electroporation was performed. The correct amount of hygromycin B to use for selection of targeted cells after electroporation with WTV2 was determined.

Due to the isogenic nature of the targeting vector, a high level of success was achieved in the two electroporations performed. Approximately 30% of surviving clones were found to have the targeted genotype with both the Wilms' internal and external probes. The continuing presence of the neomycin and hygromycin resistance sequences from WTV1 and WTV2 was demonstrated using probes specific to these sequences. A high proportion of cells in these sequentially targeted clones was found to contain the correct number of chromosomes.

Sequencing of the second targeting vector revealed the nature of the second mutation. The insertion of hygromycin resistance sequences at this point in exon 9 of *Wt1* was found to produce a similar, although not identical, truncation to the first. This also has the desired effect of producing a mutant form of WT1 that functionally lacks the last two zinc fingers.

Contrary to results obtained during analysis of the first targeting event (Patek *et al.*, 1999), ES cells of none of the genotypes studied, including wild-type, were found to express detectable *Wt1* by Northern or Western analysis. No difference in the sensitivity of the techniques used in the two cases could explain this, as the same experimental protocols were used each time. The only possible explanation is that the wild-type and DDS heterozygous cells analysed in the first case contained a proportion of differentiated cells, or had been grown in other conditions which promoted *Wt1* expression. As treatment of ES cells with retinoic acid was known to upregulate *Wt1* expression (Scharnhorst *et al.*, 1997), it was decided to differentiate the cells in this manner in order to obtain the desired *Wt1* signal.

The optimum level of all-trans retinoic acid required to induce *Wt1* expression for further experiments was determined by differentiating cells for 5 days

with different RA concentrations. A concentration of 5μM was chosen, which led to WT1 protein being detectable by Western analysis. It had previously been demonstrated using high G418 DDS homozygous cells that cells with two alleles producing proteins with truncations in ZF3 of WT1 contain no WT1 protein (C. Miles, personal communication) despite presence of mutant *Wt1* mRNA (Patek *et al.*, 1999). It unfortunately was not possible to confirm that this was also true of the compound heterozygous cells, as Northern analysis of these cells was unsuccessful. A suggested mechanism for this could be decreased mutant protein stability, as the putative WT1 nuclear localisation signal is not affected by the truncation (Bruening *et al.*, 1996), nor is WT1 protein found in the cytoplasmic fraction of these cells. The lower than expected level of the mutant forms of WT1 in the DDS heterozygote are still higher than WT1 protein levels in the compound heterozygote. This could be due to the presence of wild-type WT1 in the same cell stabilising the mutant in some way (Patek *et al.*, 1999), possibly through the formation of complexes, as WT1 is known to be able to self-associate via the N-terminal domain, which is still intact in DDS mutant protein (Holmes *et al.*, 1997).

When examining both wild-type and DDS heterozygous cells by immunofluorescence analysis, it was found that the vast majority of cells expressed WT1 after 4 days of treatment with 5μM retinoic acid. In other words, the WT1 signal produced after retinoic acid differentiation is not just due to certain cells differentiating along particular pathways and expressing WT1 in abundance, but a general effect on all cells. The expression levels of WT1 protein in wild-type and DDS heterozygous differentiated cells do not seem to differ significantly, bearing in mind that expression from the wild-type allele is normal and expression from the

mutant allele highly reduced. This is in contrast to a result published by Yang *et al.* (1999), who examined WT1 protein expression in the podocytes of DDS patients and found a much more significant decrease in this expression as compared with normal kidneys. This may be a suggestion that the situation in differentiated ES cells containing a DDS mutation may not be a true reflection of the *in vivo* situation. However, the study by Yang *et al.* (1999) was performed on biopsy specimens from patients already diagnosed with DDS, and the lengths of time between the first symptoms of nephropathy and end-stage renal failure in this study are short (median = 0.6 years). This could mean that the disappearance of WT1 protein does not occur until a fairly late stage in the progression of DDS and that the results from the ES cells are representative of an earlier stage of the *in vivo* situation.

The induction of *Wt1* expression was first seen after 36 hours of exposure to retinoic acid and was universal after 48 hours. This was accompanied by an alteration in cell morphology away from an ES cell-like appearance. This, and the length of time taken for WT1 protein to appear, suggests that induction of *Wt1* by retinoic acid is due to the resulting differentiated state of the cells, rather than a direct transcriptional effect of retinoic acid on the *Wt1* gene. This is supported by the finding that after differentiation with retinoic acid for 4 days, followed by its removal, ES cells continue to express *Wt1* for at least 10 days. If the retinoic acid were acting directly on *Wt1* transcription, WT1 protein would be expected to disappear upon its removal. Other groups examining effects of RA on WT1 expression in different cell types have found evidence which also suggests that RA does not act directly on *Wt1*. Scharnhorst *et al.* (1997) found a delay in RA-induced *Wt1* expression in ES and P19 embryonal carcinoma cells similar to that found here.

HL60 cells show a down-regulation of *Wt1* when induced to differentiate using RA, and K562 cells do not differentiate in response to RA, and no change is seen in *Wt1* expression upon its addition (Sekiya *et al.*, 1994). This would indicate that RA does not only not act directly on *Wt1* expression, but does not always even induce it indirectly when added to cells. It strongly suggests that the observed *Wt1* expression is merely the result of the nature of the cellular differentiation induced, which will of course vary depending on the nature of the starting cells.

The cell type or types produced from ES cells by the addition of RA in this experiment have not been determined. It does seem strange that, despite the variations in morphology seen in the differentiated cultures, the vast majority of these cells give a signal with WT1 antibody. *Wt1* expression during development is far from ubiquitous, with expression confined to specific developing organs, such as the kidney, at particular developmental stages (Armstrong *et al.*, 1992). Most of the tissues in which *Wt1* is found to be expressed are mesodermally derived, with the exception of some ectodermally derived regions of the central nervous system (Armstrong *et al.*, 1992). Not all mesodermally and ectodermally derived tissues express *Wt1*, however, so the control of *Wt1* expression involves far more specific mechanisms than simply the production of a particular cell type. This makes it extremely unlikely that all the cell types produced during differentiation of ES cells with retinoic acid are those which normally express *Wt1*. It would be possible to study in more detail the cell types produced by RA-differentiation of ES cells to establish the nature(s) of the cells that are shown here to express *Wt1*. This could be done through immunological techniques to look for the presence of markers of particular cell lineages, or through gene expression analysis (see section 6.1).

Denys-Drash syndrome type proteins have been shown to colocalise with cellular splicing factors in a similar manner to +KTS wild-type WT1 isoforms (Larsson *et al.*, 1995). DDS mutant proteins are also suggested to alter the function of the wild-type protein in a heterozygous cell through protein-protein interaction (Reddy *et al.*, 1995) and affect the subnuclear localisation of wild-type WT1 protein (Englert *et al.*, 1995). This suggests that binding between mutant and wild-type protein in a DDS cell could lead to the abnormal localisation of wild-type –KTS isoforms to the splicing compartment, preventing transcriptional activity. Wild-type and DDS heterozygous cells were examined using immunofluorescence to determine whether any abnormal localisation of the wild-type WT1 protein could be detected in the heterozygous cells. Two WT1 antibodies were used: the C-terminal antibody C-19 and the N-terminal antibody 6F-H2. The former will only detect the wild-type protein, as the epitope it recognises is found in the region of the protein encoded by exon 10, and is therefore missing from the truncated protein. 6F-H2 will recognise both mutant and wild-type WT1. No difference in expression pattern was seen with either antibody between the mutant and wild-type cells, suggesting that the presence of DDS mutant protein does not significantly affect the localisation of wild-type WT1 protein also present. The study by Englert *et al.* (1995), in which mutant WT1 was shown to alter the subnuclear localisation of wild-type WT1, used transient transfection of these proteins into a cell and only used one isoform at a time. This use of exogenous WT1 protein at non-physiological levels means that the experiment does not accurately reflect the *in vivo* situation. My use of endogenous WT1 protein, expressing all the different isoforms, may mean that subtle differences in the

localisation of some of the isoforms may have been hidden, whereas these were magnified in the study of Englert *et al.*

It is not known whether the colocalisation of some WT1 isoforms with the cellular splicing machinery is due to a function of WT1 protein in splicing or just a sequestration of non DNA-binding isoforms in clusters. It has been suggested that WT1 could be involved in splicing (Larsson *et al.*, 1995; Ladomery *et al.*, 1999). In this case, the presence of DDS mutant protein in a cell could affect *Wt1* RNA splicing and result in an altered ratio of WT1 isoforms. An alteration in these ratios is implicated in diseases such as Frasier syndrome (Barbaux *et al.*, 1997; Klamt *et al.*, 1998). The ratios of the various WT1 isoforms have been found to vary in a species-specific, tissue-specific and developmental stage-specific manner (Renshaw *et al.*, 1997), but the differentiated progeny of ES cells, as studied here, had not been investigated. RT-PCR analysis was used to examine the relative proportions of the RNA species giving rise to the different WT1 isoforms for two purposes: (1.) To determine whether the isoform ratios in wild-type differentiated ES cells were similar to those previously reported in other cell types and (2.) To see whether these isoform ratios differed according to genotype in DDS mutant cells.

The ratios of the different *Wt1* splice isoforms differing by the presence or absence of the exon 5 insert were determined by RT-PCR analysis. These were very similar to those published by Renshaw *et al.* (1997) for a range of mouse tissues. This previous study found a variation between different tissue types, with higher levels of exon 5-containing transcripts found in kidney and testis, but the RA-differentiated ES cell cultures examined here do show morphological heterogeneity, so isoform ratios covering the full range found in mouse tissues is not unexpected.

No difference was found between the +/- exon 5 ratios that was attributable to *Wt1* genotype. This would suggest that either WT1 has no effect on its own splicing, or that the region of the WT1 protein required for this effect is not affected by the DDS truncations examined here.

The results obtained for the ratios of *Wt1* mRNA transcripts possessing and the lacking the KTS alternatively spliced region in wild-type ES cell differentiated progeny, on the other hand, differed from previously published findings. These results were consistent between the cell lines examined, ranging from 0.71-0.91:1, but are very different from the 3.3:1 ratios obtained by Haber *et al.* (1991). Simms *et al.* (1995) and Renshaw *et al.* (1997) found +/- KTS ratios closer to 1:1, but these also had the isoforms containing the KTS insert in the majority, whereas the present study found a higher level of isoforms lacking this region. Although Renshaw *et al.* (1997) found no developmental stage-specific differences in these isoform ratios, the only foetal tissue studied was human kidney, which does not rule out the possibility that mouse foetal tissues express different ratios, more consistent than those found for ES cells in the present study.

The above results indicate that cells of the desired genotype were produced by the targeted strategy utilised. These cells contain less WT1 protein than expected, although how this relates to mRNA levels could not be determined. If these compound heterozygous cells do contain a higher level of *Wt1* mRNA than would account for the levels of protein observed, as was previously found in high G418 homozygous cells, some kind of post-translational mechanism is believed to be in action, possibly through protein stability. ES cells were found to be induced to express *Wt1* upon differentiation with all-trans retinoic acid, and the results obtained

using immunofluorescence analysis suggest that this is more likely to operate through the differentiation of the cells, rather than a direct transcriptional activation of *Wt1* by retinoic acid. Other immunofluorescence work revealed that presence of DDS mutant WT1 protein in a cell does not appear to alter the subnuclear localisation of wild-type WT1 protein also present. The presence of DDS mutant protein does not alter the relative proportions of *Wt1* mRNA species possessing and lacking the exon 5 alternatively spliced region, which were found to be very similar to the proportions of these isoforms previously published for other cell types. The proportions of +/- KTS isoforms could only be determined for wild-type cells and embryoid bodies, and were found to agree with some previously published results and differ from others.

5. Comparison of wild-type and *Wt1* mutant ES cells by differentiation into embryoid bodies.

When grown in the absence of LIF and allowed to form three-dimensional structures, ES cells will differentiate into embryoid bodies (EBs). These contain cells from all three embryonic germ layers (ectoderm, mesoderm and endoderm) (Doetschman *et al.*, 1985). EBs have proved to be a useful tool for the examination of early developmental processes. They have been allowed to differentiate over a period of time and used to study the temporal expression patterns of various genes, for example, muscle isoactin (Ng *et al.*, 1997). The effects of overexpression of particular genes in EBs has been examined, including the effects of *m-twist* expression on myogenic differentiation, and hepatocyte nuclear factor 3 on endodermal differentiation (Rohwedel *et al.*, 1995; Levinson-Dushnik and Benvenisty, 1997). ES cells null for the desmin gene have been shown through EB production to be unable to differentiate into smooth or skeletal muscle (Weitzer *et al.*, 1995).

Systems have been established for the promotion of the development of particular lineages within EBs. These involve the culture of the EBs in medium containing specifically tailored combinations of growth factors. For example, one can choose to produce outgrowths from embryoid bodies consisting predominantly of neuronal, myogenic or haematopoietic lineages (Bain *et al.*, 1994; Drab *et al.*, 1997; Wiles and Keller, 1991). Four day old EBs will produce many cells with neuronal properties when differentiated for a further four days with all-trans retinoic acid then allowed to attach to a surface and form outgrowths (Bain *et al.*, 1994).

Embryoid bodies made using hanging drop culture, grown in suspension for 5 days, then allowed to attach in the presence of all-trans retinoic acid and dibutyryl-cAMP (db-cAMP) can produce up to 60% smooth muscle cells under optimum conditions (Drab *et al.*, 1997). Wiles and Keller (1991) produced EBs by culturing ES cells in methyl cellulose, then transferred the EBs to tissue culture plates and allowed them to attach. The medium was then supplemented with IL-3 to promote mast cell growth and IL-3 and M-CSF for macrophage development.

As *Wt1* is expressed early in development (mouse early E9 – Armstrong *et al.*, 1992), it was thought that embryoid body experiments might be a useful method for assessing the developmental capabilities of *Wt1* mutant ES cells. This initially just consisted of a straightforward EB differentiation in the absence of LIF, and then proceeded to include an examination of haematopoietic differentiation in wild-type and *Wt1* mutant embryoid bodies.

5.1 Use of RT-PCR analysis to examine *Wt1* expression in embryoid body time courses

RT-PCR analysis was used to demonstrate that *Wt1* expression was occurring within embryoid bodies, so that any differences in phenotype found could be attributed to the *Wt1* mutation. It was established that undifferentiated ES cells do not express *Wt1* at levels detectable by Northern or Western analysis (see section 4.2.4), but that ES cells differentiated with 5μM retinoic acid for four days express high levels of *Wt1* (section 4.3.1).

Analysis was performed on the same cDNA samples as used in section 5.3.4, using the K447 and K448 *Wt1* primers (see section 4.4.2). These results are presented in table 5.1, and level of expression, is assigned a value on a scale ranging from – (no detectable expression) to +++ (high levels of expression).

Table 5.1: *Wt1* expression in embryoid body time courses measured using RT-PCR

Diffn. time (days)	Genotype					
	CGR8 embryoid bodies			E14 embryoid bodies		
	+/+	+/-	-/-	+/+	+/-	-/-
4	+	+++	+	-	++	+++
8	+	+	+	+	++	not done
12	+	+	+	+	+	++

+/+ wild-type

+/- heterozygote

-/- homozygote/ compound heterozygote

It can be seen from the table that *Wt1* expression was detected at all but one time point examined, for both CGR8 and E14 embryoid bodies. There appears to be no obvious relationship between levels of expression and time, but the fact that *Wt1* expression is detected at all in these time courses means that any phenotypic differences mentioned later in this chapter which vary consistently with *Wt1* genotype may well be due to the *Wt1* mutation.

5.2 *In vitro* differentiation of wild-type, DDS heterozygous and DDS compound heterozygous CGR8 ES cells into embryoid bodies

As *Wt1* may play an important role in the differentiation of several different cell types, it was decided to produce embryoid bodies from wild-type, DDS heterozygous and DDS compound heterozygous ES cells. The aim was to see whether the *Wt1* mutant cells were incapable of producing any particular lineages, or whether an excess of a particular cell type could be detected, for example muscle cells, as myogenic differentiation is believed to be promoted in the absence of WT1 (Miyagawa *et al.*, 1998).

ES cells were differentiated by the cell aggregation method (Martin and Evans, 1975; Smith and Hooper, 1987), in which cells are grown to high density in a dish, then gently broken up into clumps and allowed to differentiate into embryoid bodies in suspension in the absence of LIF. Embryoid bodies were either kept in suspension until being harvested for analysis, or removed to tissue culture dishes just after cavitation and allowed to attach to the surface of the dish and form outgrowths.

Suspension embryoid bodies were harvested at various time-points, embedded, then sectioned and stained with haematoxylin and eosin. These sections were then examined by bright field microscopy to see whether any differences in histology were apparent. Embryoid bodies that had been allowed to attach to the surface of a dish were examined by phase contrast microscopy while growing.

Suspension embryoid bodies were found to exhibit endoderm formation and cavitation (figure 5.1), and outgrowths from attached embryoid bodies showed a range of cell types, including beating cardiomyocytes (figure 5.2). No difference was

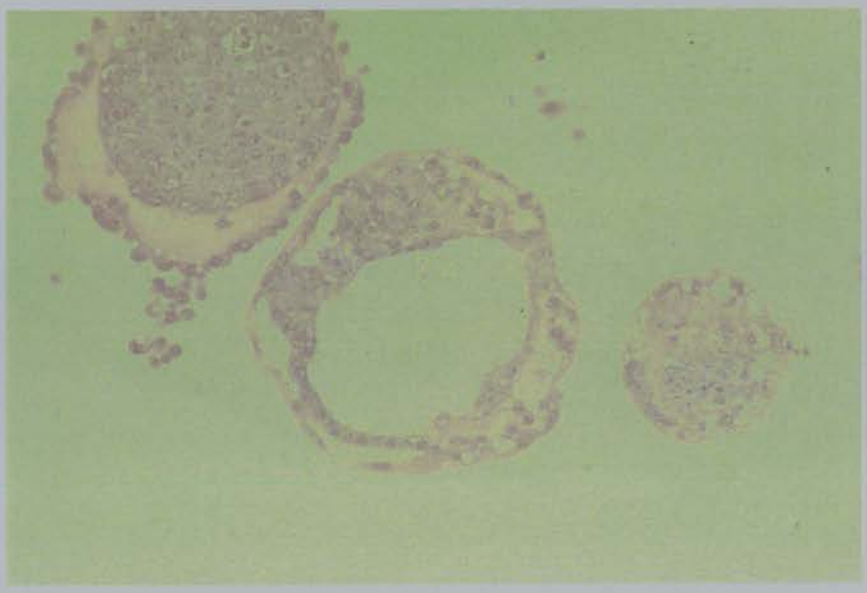


Figure 5.1 Section of suspension embryoid bodies

Section of H&E stained CGR8 DDS compound heterozygous embryoid bodies after 14 days differentiation in suspension. Parietal and visceral endoderm and cavitation can be observed.

Magnification x200.

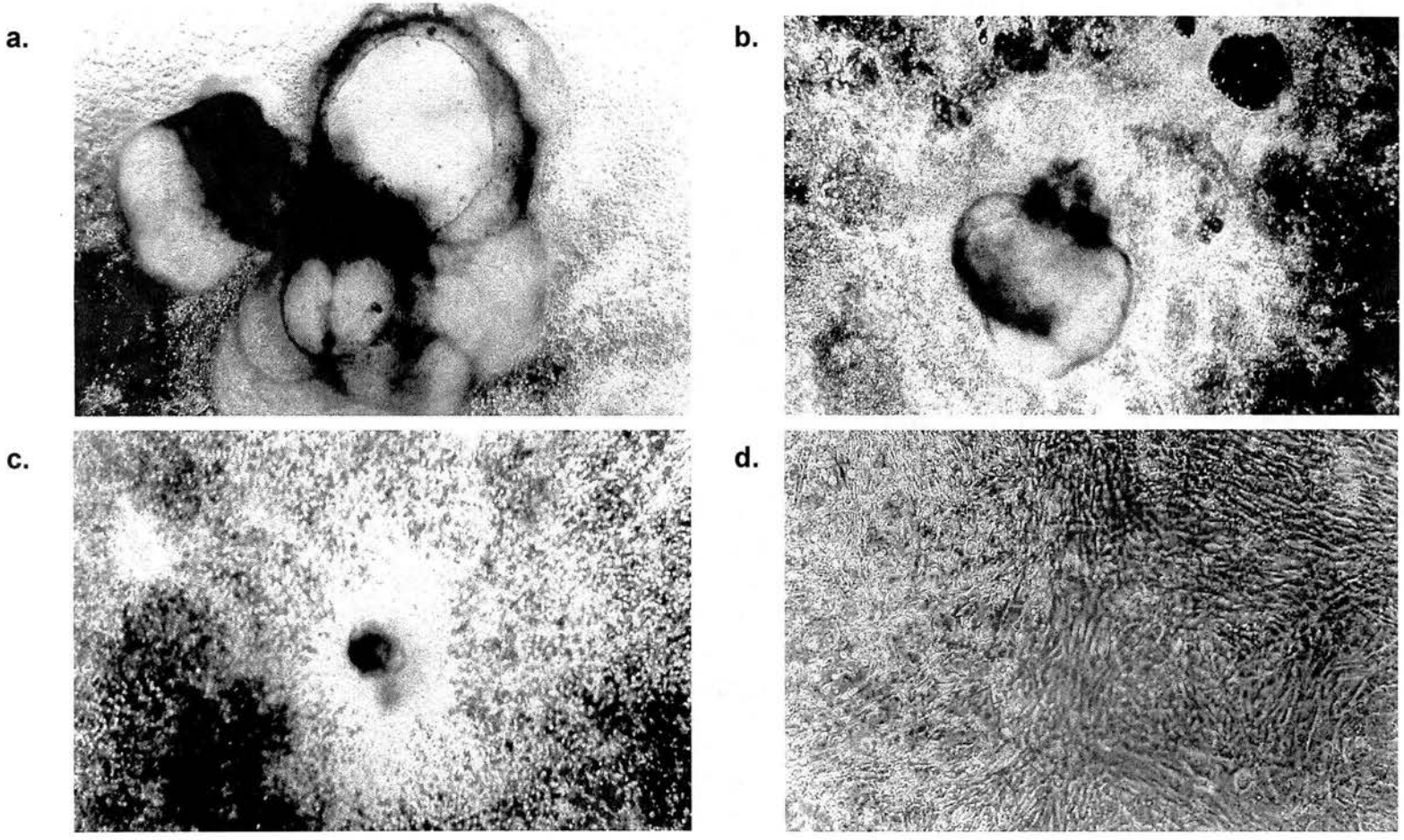


Figure 5.2 Embryoid bodies allowed to differentiate whilst attached to the surface of a dish

Wild-type embryoid bodies and their outgrowths after attachment to a surface and continued differentiation for 14 days. Photographs **a** and **b** show cysts which have formed from embryoid bodies, surrounded by a monolayer of differentiated cells. **a, b & c** – x40, **d** – x200

seen between the three genotypes of embryoid body in terms of time taken for cavitation to occur or presence of beating cardiomyocytes. No cell types were obviously missing or more prevalent in mutant or wild-type embryoid bodies. This suggests that absence of wild-type WT1 protein has no gross effect on the lineages produced during embryoid body development. No blood islands were observed during these experiments, which therefore yielded no information on any role of WT1 in haematopoiesis. It would, of course, be possible to use marker genes for specific lineages to examine embryoid body development in more detail, but no starting point for this analysis was suggested by this experiment. As no apparent differences were seen, it was decided to use a more subtle approach to specifically examine *in vitro* the role of WT1 in haematopoiesis.

5.3 Investigating the role of WT1 in early haematopoiesis using the CFU-A assay and RT-PCR

The role of WT1 in haematopoiesis remains unclear. As described in section 1.7, it is known that *WT1* expression occurs in the population of bone marrow cells believed to contain the HSC and is then down-regulated (Baird and Simmons, 1997). This down-regulation is accompanied by cellular differentiation, suggesting that it could be some kind of switch, required to allow differentiation to proceed. Indeed, the expression of antisense *WT1* oligonucleotides in proliferating leukaemic cells induces them to differentiate (Yamagami *et al.*, 1996), and expression of WT1 in monoblastic cell line U937 prevents differentiation, even in the presence of inducing agents (Svedberg *et al.*, 1998). This being the case, the importance of *WT1* in the

haematopoietic differentiation process must be considered. Is *WT1* expression in this period of early haematopoietic development crucial for later development to occur?

The definitive test for haematopoietic potential is through long-term repopulation of a lethally-irradiated recipient mouse (Moore and Metcalf, 1970), but *in vitro* assays are also available which can give an idea of the ability of cells to generate haematopoietic lineages (Johnson *et al.*, 1980; Humphries *et al.*, 1979; Pragnell *et al.*, 1988). These generally involve the use of semi-solid media, such as methylcellulose or agar, containing specific haematopoietic growth factors, or conditioned medium known to contain such growth factors. Cells such as bone marrow or ES cells, or embryoid bodies, are grown in this environment and the morphologies of any colonies formed are examined by phase contrast microscopy (Pragnell *et al.*, 1988; Hole *et al.*, 1996). The growth factors used in the assay determine from which subset of haematopoietic progenitors colonies will form. Such assays include those for high proliferative potential colony-forming cells (HPP-CFC), granulocyte-macrophage colony-forming units (CFU-GM) and colony forming unit A (CFU-A). Relationship of these to one another and to the CFU-S detected by the *in vivo* spleen colony-forming assay has been determined, and a presumptive hierarchy established (figure 5.3)(Kriegler *et al.*, 1994).

5.3.1 Comparison of wild-type and *Wt1* mutant ES cells from the CGR8 and E14 lines using an *in vitro* assay for haematopoietic differentiation

As a role for WT1 has been suggested in leukaemia (see section 1.7), it was decided to compare the relative performances of the three DDS genotypes of ES cells in an *in vitro* assay to determine the potential of cells to differentiate into

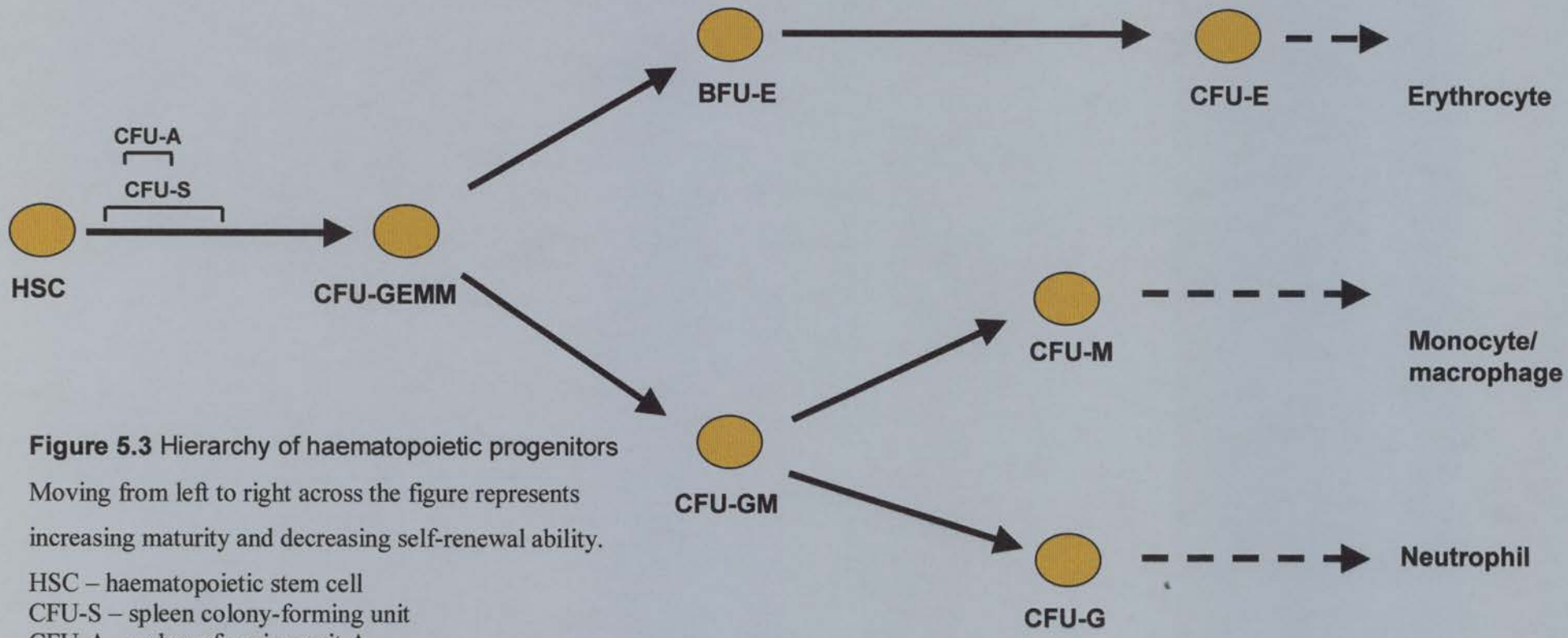


Figure 5.3 Hierarchy of haematopoietic progenitors

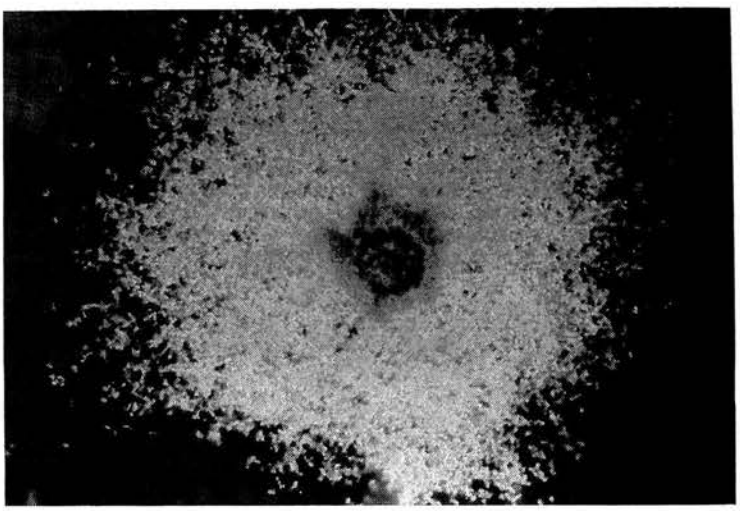
Moving from left to right across the figure represents increasing maturity and decreasing self-renewal ability.

HSC – haematopoietic stem cell
CFU-S – spleen colony-forming unit
CFU-A – colony-forming unit A
CFU-GEMM –colony-forming unit granulocyte-erythrocyte- macrophage - megakaryocyte
BFU-E –burst-forming unit erythroid
CFU-E –colony-forming unit erythroid
CFU-GM –colony-forming unit granulocyte-macrophage
CFU-G –colony-forming unit granulocyte
CFU-M –colony-forming unit macrophage

haematopoietic progenitors. The CFU-A assay was chosen to compare the abilities of different DDS genotypes to differentiate into haematopoietic cells because it examines a very early stage of haematopoietic development, before the lymphoid/myeloid split (see figure 5.3), and as *WT1* mutations have been identified in leukaemias originating from both these lineages, the role of WT1 in normal haematopoiesis is likely to occur at an early stage (King-Underwood and Pritchard-Jones, 1998). Another reason for the use of this assay is that the behaviour of wild-type ES cells in the CFU-A assay has previously been well characterised (Hole *et al.*, 1996). It has been demonstrated that wild-type embryoid bodies will produce recognisable haematopoietic cells even in the absence of externally applied growth factors (Wiles and Keller, 1991). The CFU-A assay uses haematopoietic growth factors to encourage the growth of haematopoietic colonies from embryoid bodies (Hole *et al.*, 1996). The growth of a colony indicates the presence of at least one haematopoietic progenitor within the embryoid body from which it grew. Positive colonies are at least 1mm in diameter, and consist of mixed myeloid lineages, predominantly macrophages (figure 5.4)(Hole *et al.*, 1996).

Embryoid bodies were made using the hanging drop method (see section 3.1.11) from wild-type, DDS heterozygous and DDS compound heterozygous CGR8 ES cells. This method produces embryoid bodies of a uniform size, allowing comparisons between different genotypes. These were then allowed to differentiate in the absence of LIF for periods ranging from 2 to 18 days. The embryoid bodies were then plated into the assay and grown in a 5% CO_2/ 10% O_2 incubator in the presence of conditioned medium containing GM-CSF, CSF-1 and SCF for 7 to 14 days, at

a.



b.

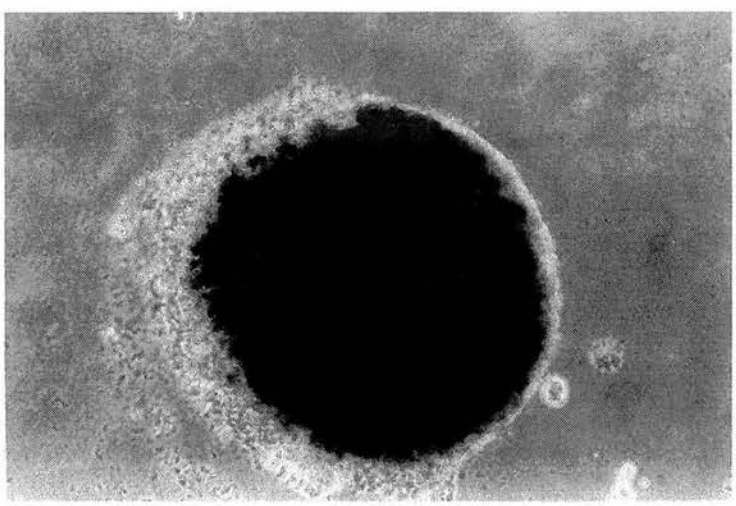


Figure 5.4 CFU-A positive and negative colonies.

The CFU-A assay was set up as described in the text. After 10 days, the assay was scored, and colonies were photographed using a light microscope.

a. CFU-A positive colony (x40). A 'halo', consisting of cells of mixed myeloid lineages, has grown outwards from the original embryoid body, still seen in the centre.

b. CFU-A negative colony (x100). This colony contained no haematopoietic precursors of a suitable maturity to respond to the growth factors present in the assay. No change has occurred to the embryoid body.

which point the dishes were scored to determine the percentage of embryoid bodies initially plated which had given rise to a CFU-A positive colony.

It is already known that wild-type embryoid bodies, regardless of their ES cell line of origin, follow a specific temporal pattern in the CFU-A assay (Hole *et al.*, 1996). Very few colonies are produced from EBs which have been differentiated for less than four days. After this, the proportion of CFU-A positive colonies rises to a peak of approximately 40-60% somewhere between days six and eight, which then tails off. The exact percentage of positive colonies at the peak of the graph and the timing of this peak vary between experimental runs and cell lines, but the basic pattern remains the same. This pattern suggests that it takes at least four days of culture in the absence of LIF to produce cells of haematopoietic lineages, or at least to produce haematopoietic cells of sufficient maturity to respond to the growth factors present in the assay and produce a colony. The average length of time taken to produce such cells, however, is six to eight days. The fact that the proportion of positive colonies decreases after the peak suggests that there is a limited temporal window for the production of haematopoietic progenitors leading to CFU-A colonies. After this time, the haematopoietic cells present in the EBs are too mature to produce CFU-A colonies, and either produce smaller, CFU-GM-like, colonies, or none at all (Hole *et al.*, 1996).

Wild-type ES cells used in this experiment came from both the CGR8 and E14 cell lines. A single experiment using the CGR8 cell line and mutants derived from it is shown in figure 5.5, and summary data for all CGR8 experiments presented in table 5.2. The CGR8 EBs were consistently shown to peak on days 6 to 8 (table 5.2), with a peak proportion of positive colonies of around 30-40%. The

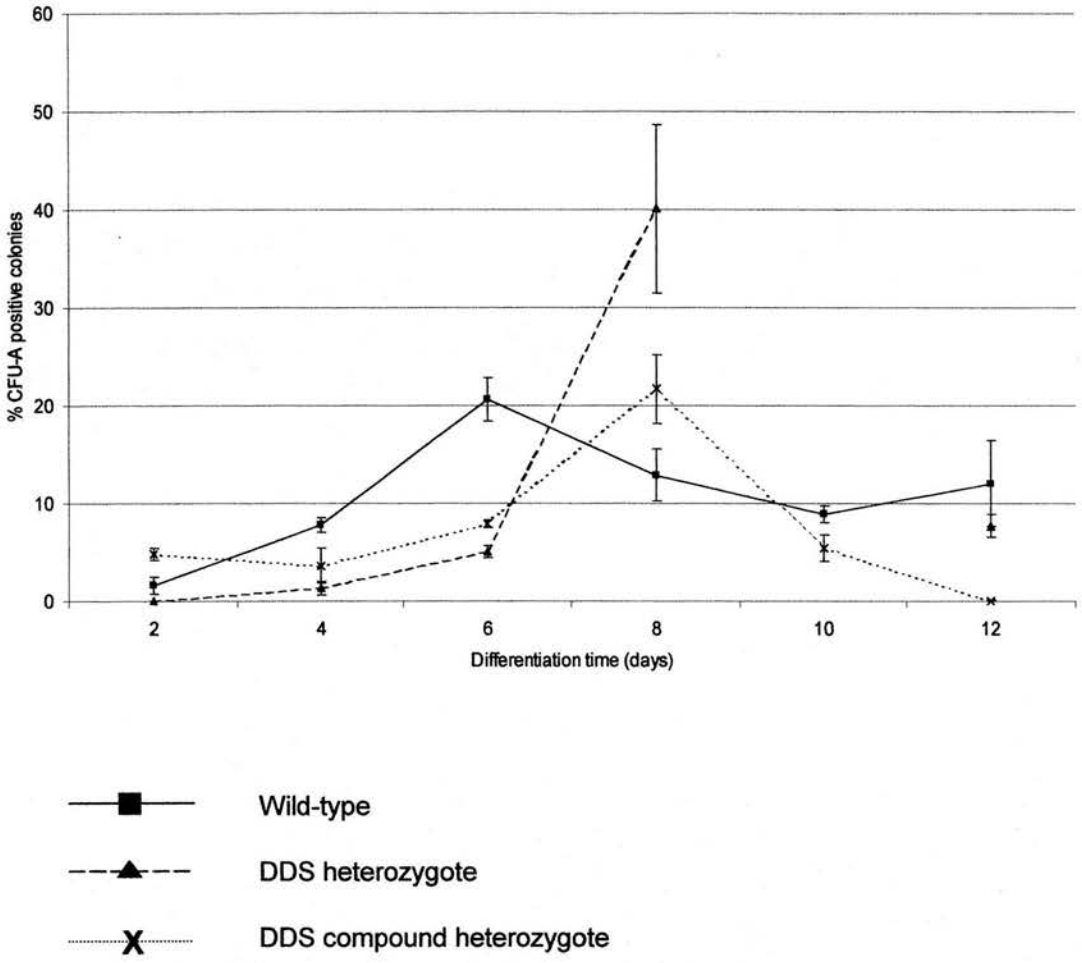


Figure 5.5 CFU-A activity in CGR8 embryoid bodies

Cell aggregates were prepared using hanging-drop culture, then differentiated in the absence of LIF for times ranging from 2 to 14 days. The resulting embryoid bodies were then plated into semi- solid agar containing the growth factors GM-CSF, CSF-1 and SCF and incubated for 7 days. Colonies over 1mm in diameter were scored as being CFU-A positive. This is presented as a proportion of the total embryoid bodies plated. Both DDS heterozygous and DDS compound heterozygous embryoid bodies are seen here to take longer than the wild-types to reach their maximum colony production.

Data from experiment 4 presented in table 5.1.

Error bars show standard errors.

peaks in E14 experiments varied from day 6 to day 12, with a tendency towards the later time points (table 5.3).

In all experiments, the DDS compound heterozygous CGR8 EBs took longer than the wild-type EBs to reach their peak haematopoietic activity (figure 5.5, table 5.2). This delay varied from two to more than four days relative to the wild-type cells. DDS heterozygous CGR8 cells also showed a delay, ranging from two to four days later than the wild-type cells (table 5.2). This peak was usually seen to coincide with the peak in the compound heterozygous cells, but on occasion fell between the wild-type and compound heterozygous peaks. As time points in this study are two days apart, there is not sufficient resolution to detect the difference, if any, between these two genotypes.

The height of the peak (maximum proportion of embryoid bodies producing a CFU-A positive colony) varies considerably between the different experimental runs. There is no consistent difference between the different genotypes regarding the height of this peak, suggesting that, once *Wt1* mutant embryoid bodies begin producing CFU-A progenitors, this occurs at the same frequency as it does in wild-type embryoid bodies.

Table 5.2: Summary of CFU-A assay results for CGR8 cell line and its derivatives

Experiment number	Day of differentiation at which CFU-A peak reached, with height of peak in brackets		
	+/+	+/-	-/-
1	8 (24%)	10 (36%)	14+ (37%+)
2	8-10 (33%+)	12 (5%)	12 (3%)
3	8 (40%)	No colonies	12 (15%)
4	6 (20%)	8+ (40%+)	8 (22%)

A second heterozygous clone was also used in each experiment as a control for passage number, as the compound heterozygous cells have been through many more passages than the wild-type cells, due to two targeting and selection procedures. These control clones were heterozygous cells that had undergone the second electroporation and survived the selection, but were found to have not undergone homologous recombination at the wild-type *Wt1* allele. These cells had undergone approximately the same number of passages as the compound heterozygous cells, but had the same genotype as the heterozygotes, so could be used as a control to determine whether any effects seen were due to genotype or just to increased passage number. Unfortunately, these cells failed to produce more than a handful of colonies in most of the experiments, so it is hard to say when they actually peak. However, as the heterozygote and compound heterozygote usually peaked at the same time anyway, it is unlikely that passage number had any effect here, making the control unnecessary.

The E14 ES cells used in this study generally formed fewer CFU-A positive colonies than the CGR8 cells. Three genotypes were used: wild-type, DDS heterozygote, and DDS homozygote (made by high G418 selection). The results obtained from the CFU-A assay comparison of the E14 cells were consistent with those obtained from the CGR8 cells (table 5.3). The results for only wild-type and DDS homozygous cells are summarised in table 5.3, as DDS heterozygous cells failed to produce colonies in most of the experiments. The height of the peak in DDS homozygous embryoid bodies is considerably lower than that in wild-type embryoid bodies, but as this effect was not seen in CGR8 experiments, it is unlikely to be an effect of the *Wt1* mutation.

Table 5.3: Summary of CFU-A assay results for wild-type E14 cell line (+/+) and its DDS homozygous derivative (-/-)

Experiment number	Day of differentiation at which CFU-A peak reached, with height of peak in brackets	
	+/+	-/-
1	8-10 (13%)	12 (21%)
2	12 (26%)	12 (7%)
3	6-8 (27%)	12+ (5%+)
4	8-10 (81%)	12+ (26%+)
5	10 (59%)	10 (21%)

An *in vitro* assay such as this can only provide an indication of the potential of cells to undergo haematopoietic development. The precise natures of the cell

types capable of forming a colony in this assay are unknown, as are other factors, such as the relationship of *in vivo* primitive and definitive haematopoiesis (see section 1.3) to the results seen here. Are both of these represented in the typical CFU-A curve, or only one? If both are represented, how would the graph change if the effects of one of these processes were removed? It could be the case that absence of WT1 completely blocks all primitive haematopoiesis, for example, and it is this lack which causes the observed delay on the graph. As previously mentioned, the definitive test for the potential of cells from embryoid bodies of different stages to form haematopoietic lineages is to examine their ability to achieve long-term repopulation of the haematopoietic system of a lethally-irradiated mouse (Moore and Metcalf, 1970).

5.3.2 Staining of embryoid bodies produced from wild-type, DDS heterozygous and DDS compound heterozygous ES cells for the presence of haem

Another drawback to the use of the CFU-A assay in the study of haematopoietic potential is that it relies on the response of haematopoietic cells within the embryoid bodies to specific growth factors in the conditioned medium. This means that a failure to produce colonies in the assay could be due to a blocked response to the growth factors, rather than a failure of haematopoiesis itself. So, the effect observed in the *Wt1* mutant embryoid bodies could be due to a failure of the cells to respond to GM-CSF, CSF-1 and SCF, not to a failure to differentiate along this pathway. This can be tested using an independent method to assess haematopoietic differentiation, one that is not reliant on growth factors.

Primitive erythropoiesis is the first haematopoietic activity seen in the yolk sac of the developing embryo (Moore and Metcalf, 1970). This being the case, markers for erythropoiesis should be detectable in a time course of embryoid bodies produced as described above. Blood islands are often observed during embryoid body development (Wiles and Keller, 1991), however none were seen here in either wild-type or *Wt1* mutant ES cells.

Cells of erythroid lineage contain haemoglobin, which can be visualised by staining with *o*-dianisidine, an analogue of benzidine (figure 5.6). This enables the proportion of EBs containing erythroid cells to be determined easily, and a comparison of erythropoietic activity between wild-type and mutant EBs over a time course could be performed. This is a method of assessing haematopoietic activity completely independent from the CFU-A assay, and the results could therefore reveal whether *Wt1* mutation has a genuine effect on haematopoiesis, or if the CFU-A results are simply due to a failure of the growth factors in the assay to act on haematopoietic cells which are actually present.

A time-course of embryoid bodies was produced, which were differentiated from 2 to 18 days. This consisted of CGR8 ES cells of two genotypes: wild-type and DDS compound heterozygote. Embryoid bodies from the time-course were plated into the CFU-A assay as usual. Excess embryoid bodies that were not required for the assay were stained for the presence of haem using *o*-dianisidine.

The proportion of embryoid bodies that stained positive for haem produced a graph very similar in appearance to that for the CFU-A assay for the same time-course (figure 5.7). The peak of the curve is again seen later for the DDS compound heterozygous cells than for the wild-types. This indicates that the delay seen in the

Figure 5.6 An embryoid body stained with *o*-dianisidine to reveal the presence of haem.

Embryoid bodies made by hanging drop culture were stained with *o*-dianisidine to reveal the presence of haem, an indicator of erythroid differentiation (see section 2.1.12). The staining can seen as red-brown spots on this embryoid body. Magnification = x200.

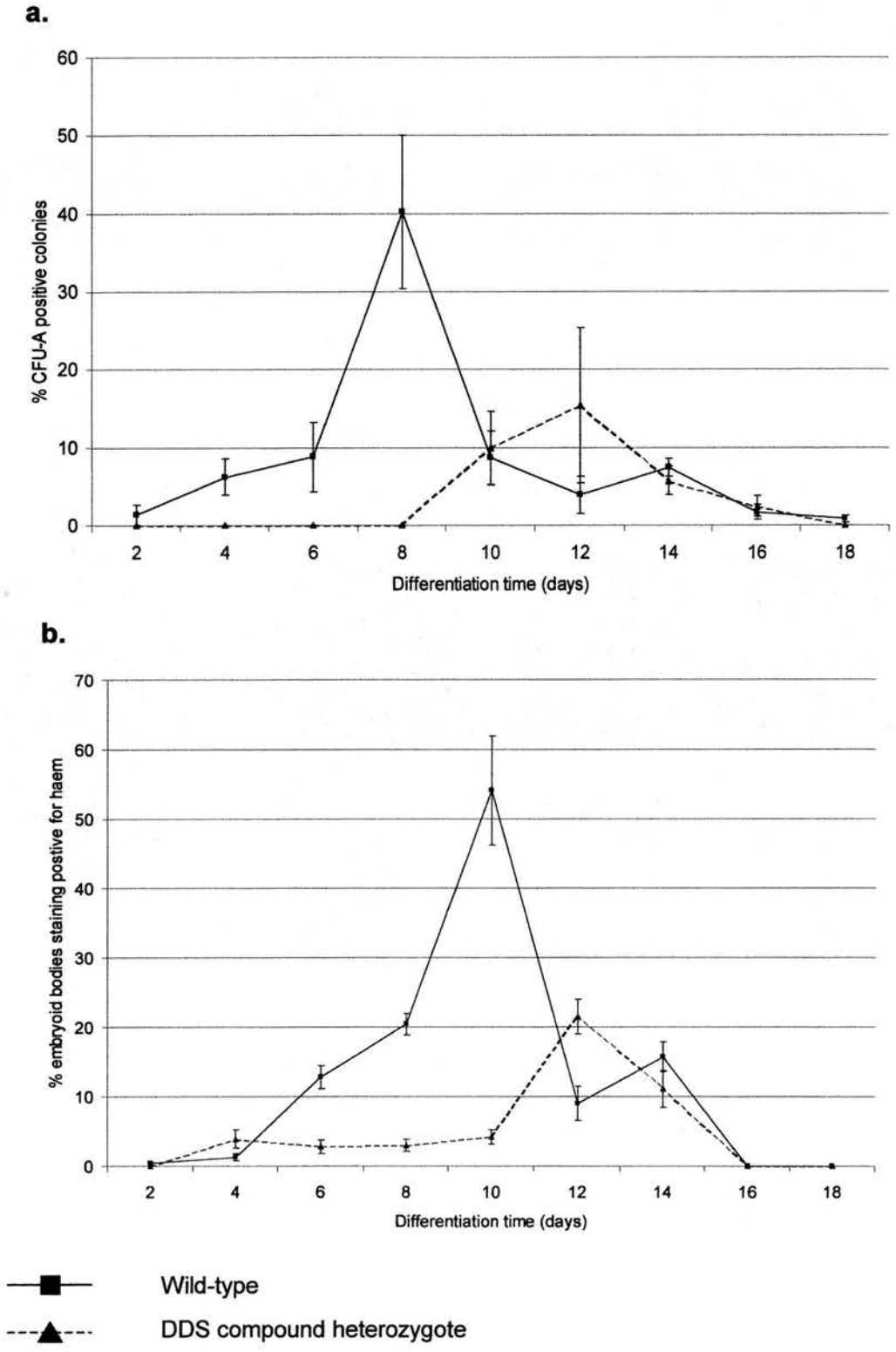
Legend to figure 5.7:

a. The embryoid body time course was produced as described in section 3.1.11, th approximately 150 embryoid bodies were plated into CFU-A dishes (section 3.1.13)

b. Excess embryoid bodies were stained using *o*-dianisidine to reveal the presence haem (section 3.1.12). The proportion of embryoid bodies showing any staining w scored.

Error bars show standard errors.

Figure 5.7 CFU-A and haem formation in the same populations of wild-type and mutant CGR8 embryoid bodies.



CFU-A assay is a real effect of the mutation on the haematopoietic differentiation pathway, rather than an interaction between the mutation and the assay. In other words, the later presence of CFU-A positive colonies in the mutant cell line is not due to an effect of the mutation on the ability of cells to react to the growth factors present in the assay, thus preventing the appearance of colonies from embryoid bodies containing haematopoietic cells at the appropriate stage of maturity.

In the wild-type EBs, the peak of erythropoietic activity, as measured by *o*-dianisidine staining, occurs 2 days later than the peak seen in the CFU-A graph for the same EBs. This is as expected, as the haematopoietic cells detected by *o*-dianisidine are more mature than CFU-A, and would therefore be expected to take longer to appear. The peaks seen in the compound heterozygous graphs, however, appear to coincide. This could suggest that once haematopoietic differentiation does begin in the mutant cells, it proceeds more rapidly than in wild-type cells, but there is evidence from the CFU-A assays that refutes this. Many of the CFU-A assays performed were scored at more than one time point. An increase in the proportion of positive colonies was seen between the earlier and later scorings (figure 5.8). This is to be expected, as more primitive precursors will take longer to produce a positive colony, and these will be detected at the later time, but not the earlier. No differences were seen in the size of this increase between mutant and wild-type embryoid bodies, indicating that there is no difference in the rate at which the haematopoietic precursors appear between the mutant and wild-type EBs.

It is interesting to note how similar the proportions of embryoid bodies displaying haematopoietic activity are when measured by two separate methods. The CFU-A assay gave proportions of 40 and 15% at the peak for the wild-type and

Legend to figure 5.8:

Embryoid bodies were plated into the CFU-A assay as before. The proportion of positive colonies for each genotype was counted after 7 days of incubation. The dishes were then replaced into the incubator and left for another 7 days before being scored again.

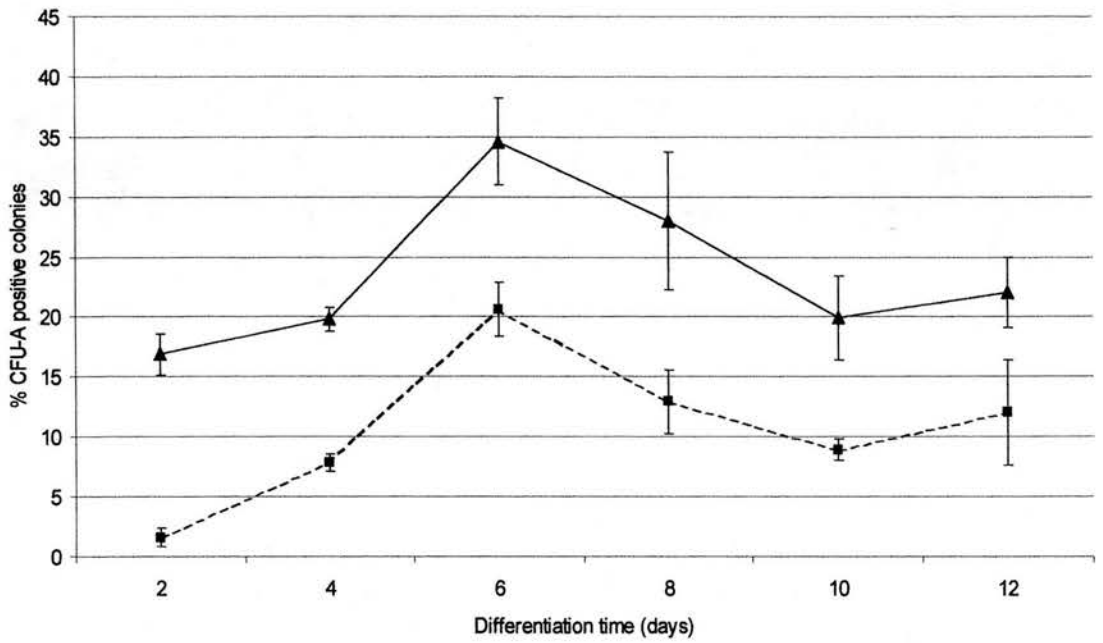
a. Wild-type embryoid bodies

b. DDS compound heterozygous embryoid bodies

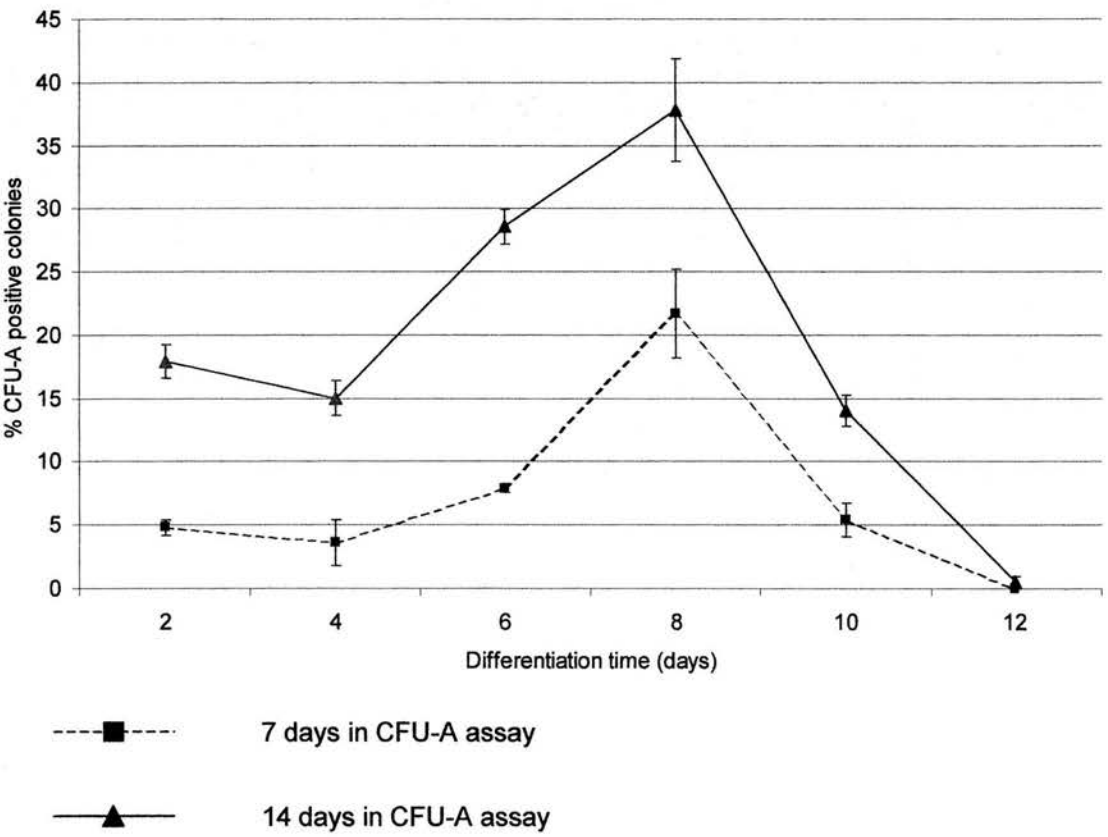
Error bars represent standard errors.

Figure 5.8 Comparison of maturity of haematopoietic progenitor cells in wild-type and compound heterozygous CGR8 embryoid bodies.

a.



b.



compound heterozygote respectively, while *o*-dianisidine staining revealed 54 and 21%. This does confirm that these two methods are measuring related effects, albeit by different approaches

The observation that the proportion of EBs containing erythroid cells peaks then falls is possibly unexpected, as erythrocytes continue to contain haem throughout their life span. The life span of an enucleated definitive mouse erythrocyte is 42-56 days (Loeffler *et al.*, 1989), but as the stage of erythropoiesis represented here is likely to occur very early in development, the erythroid cells produced here may be nucleated cells, of the type produced during primitive haematopoiesis, about which little is known. This loss of erythroid cells is consistent with that found by Wiles and Keller (1991), however, who observed formation of blood islands in embryoid bodies, followed by their disappearance after 10 days of culture. This may reflect a one-off, rather than continuous, pattern of erythroid differentiation, in which differentiation only commences up to a certain time, then cells mature and die and are no longer observed.

5.3.3 Replating of CFU-A colonies from wild-type and DDS compound heterozygous embryoid bodies

In order to gain a better understanding of the mechanisms by which the *Wt1* mutations exert their effects, it was decided to replate primary CFU-A colonies to examine any effects of these mutations on the self-renewal potential of individual colonies.

An embryoid body plated into the CFU-A assay will produce a positive colony whether it contains one CFU-A cell or several. Replating dissociated colonies

from the CFU-A assay will reveal how many CFU-A cells were present in the primary colony. This could be important, as it could give an insight into the mechanism by which *Wt1* mutations cause a delay in differentiation in this pathway.

If the lack of WT1 causes cells to be held at an early stage of haematopoietic differentiation (see figure 5.9), leading to increased self-replication, rather than differentiation, primary colonies from the earlier stages of the time-course should contain a higher proportion of cells capable of producing secondary colonies after disaggregation. Thus, more secondary colonies would be produced per primary colony replated. If, however, the lack of WT1 prevents the cells from entering the haematopoietic differentiation pathway altogether for a time (see figure 5.9), there would be no apparent increase in self-renewal in the early stages, and the graph for the mutant secondary colonies would resemble that for the wild-type colonies, but shifted to the right due to the delay.

A short time-course of embryoid bodies was produced from wild-type and DDS compound heterozygous ES cells, with EBs differentiated for 4, 6 and 8 days. These were plated into a CFU-A assay, as described previously. After 7 days in the assay, the proportion of embryoid bodies producing CFU-A positive colonies was scored. The next day, individual positive colonies were picked out of the CFU-A plates, dissociated into single cells, then plated back into CFU-A dishes, each primary colony into its own dish. These dishes were replaced in the low oxygen incubator for a further 10 days, then the number of positive colonies on each dish was scored.

The initial CFU-A graph suggests that the wild-type peaks on day 4, and the compound heterozygote may be peaking on day 8 (figure 5.10). These times are both

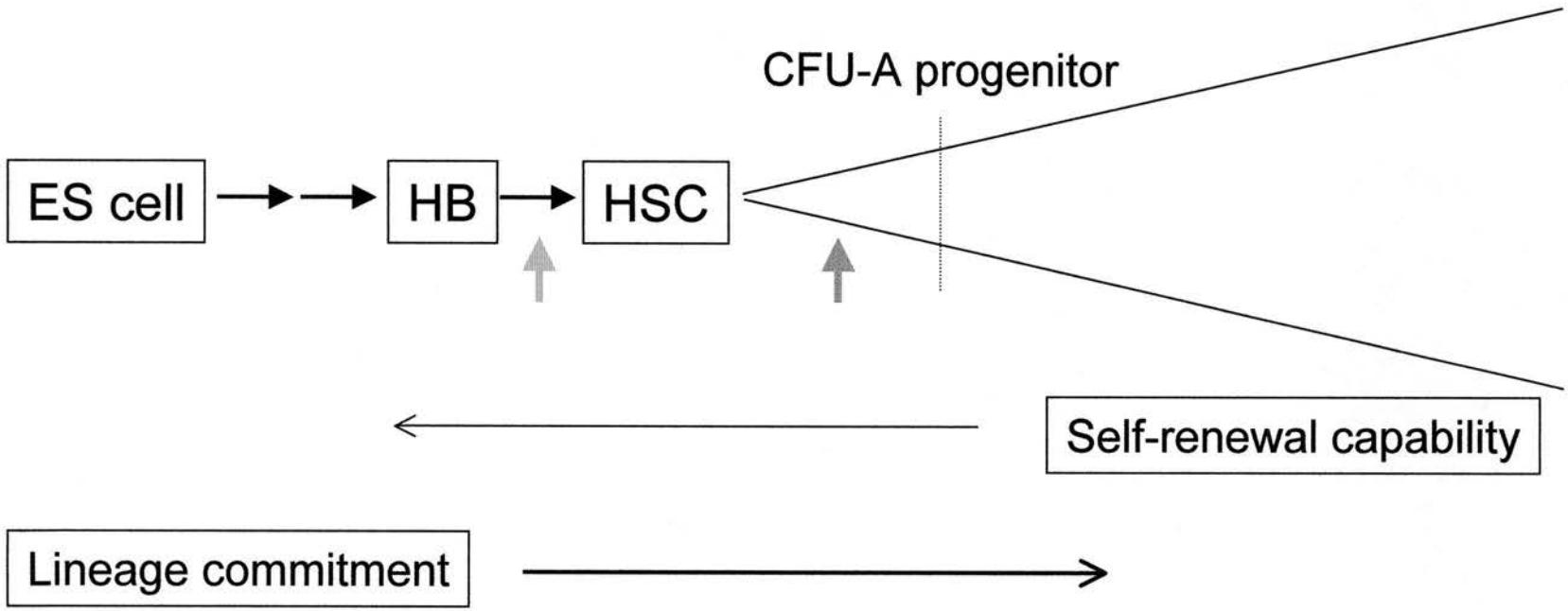


Figure 5.9 Representation of haematopoietic differentiation

The diagram represents haematopoietic differentiation starting with the ES cell and progressing through to mature, terminally differentiated blood cell. Moving from the left to the right of the diagram involves an increase in lineage commitment and a decrease in the self-renewal ability of the cells.

The arrows represent points at which mutation of *Wt1* could cause a delay in haematopoiesis, either before (red) or after (blue) the formation of the haematopoietic stem cell, and entry into the exclusively haematopoietic differentiation program, or after this point, when haematopoietic differentiation has begun.

ES – embryonic stem cell

HB – haemangioblast

HSC – haematopoietic stem cell

Legend to figure 5.10:

A time course of embryoid bodies was plated into the CFU-A assay as befo
After 8 days of incubation, the colonies formed were picked, disaggregated and repla
into the CFU-A assay in separate dishes.

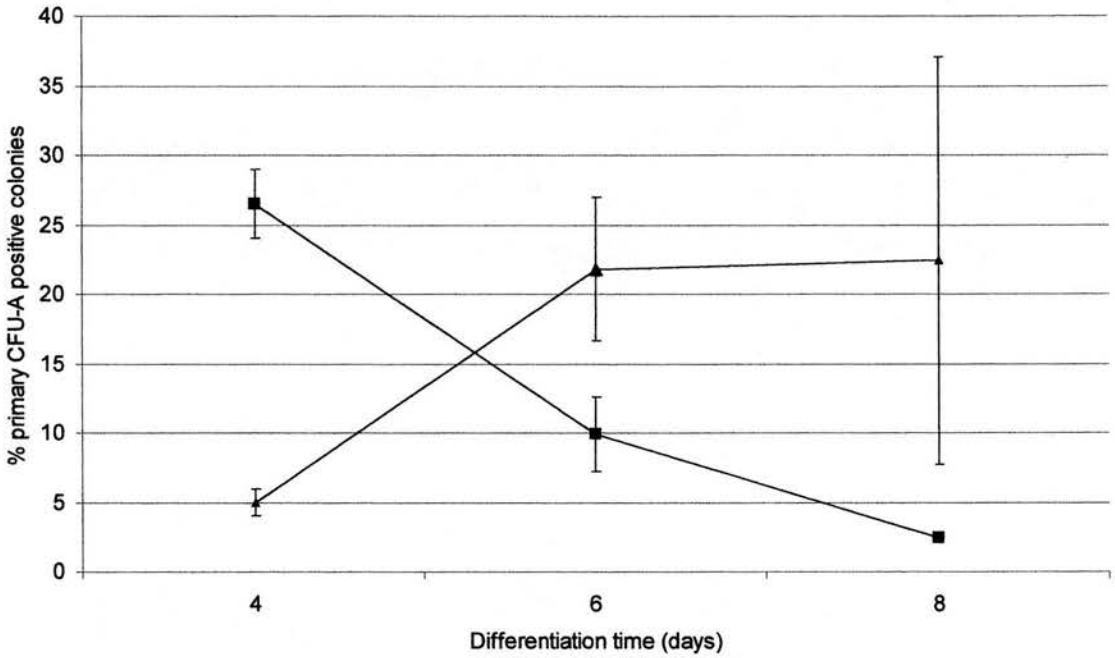
a. Initial CFU-A assay.

b. Secondary CFU-A assay.

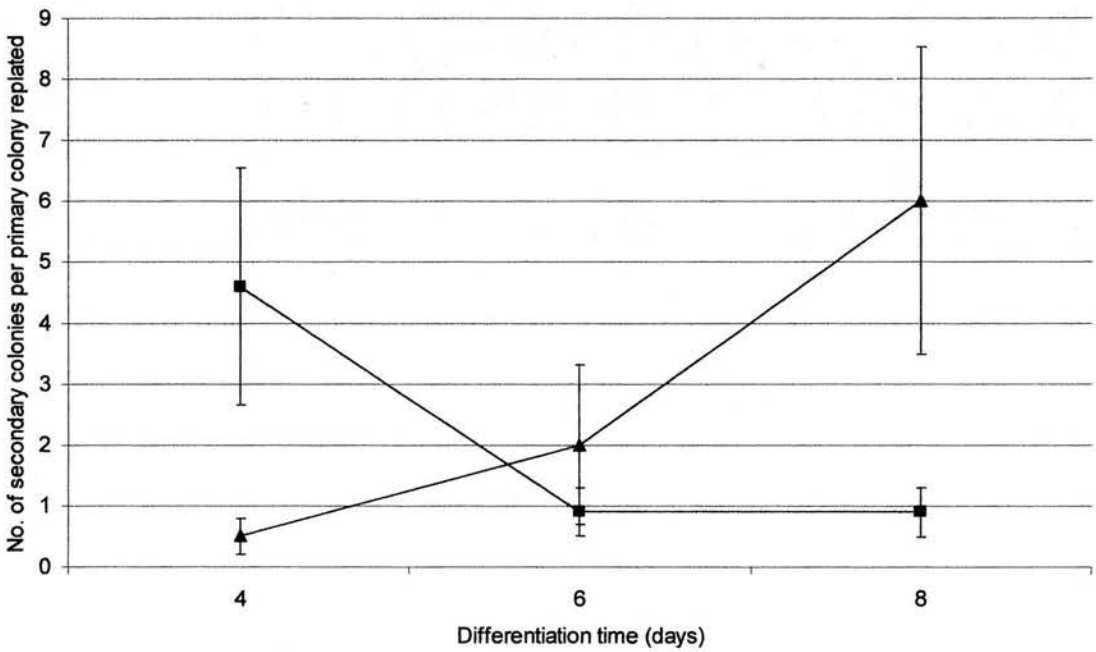
Error bars show standard errors.

Figure 5.10 Comparison of primary CFU-A positive colony formation with secondary colony formation after replating.

a.



b.



Wild-type

DDS compound heterozygote

earlier than has been found in previous experiments, but the time taken to reach the peak proportion of positive colonies is subject to variation between runs. In the wild-type embryoid bodies, the highest level of self-renewal is observed when the percentage of primary CFU-A positive colonies is highest, at 4 days of differentiation, when a large number of haematopoietic progenitors have appeared. By day 6, both the level of CFU-A positive colonies and the number of progenitors in each colony have diminished. This indicates that many of the progenitors present at day 4 have differentiated into something not detected by the assay, for example erythroid lineages, by day 6, or have matured into a myeloid cell not capable of producing a colony in this assay. The amount of self-renewal per primary colony does not alter between days 6 and 8 in wild-type cells.

In the mutant, very few CFU-A positive colonies are present at day 4, and each of these has low self-renewal ability. Both the number of primary colonies and the self-renewal potential of each colony increase by day 6, indicating an increase in both self-renewal and differentiation of progenitors into myeloid lineages. Between days 6 and 8, there is a large increase in self-renewal capacity, yet no significant change in the number of primary colonies produced.

A comparison of this pattern with that seen in the wild-type suggests that *Wt1* mutation blocks entry into the haematopoietic differentiation pathway. Unfortunately, no data were obtained from wild-type embryoid bodies before the peak level of primary colonies was reached. Even so, it is possible to see that the amount of self-renewal present in the mutant primary colonies is low at the time when the primary wild-type level is high, suggesting that these mutant embryoid bodies do not contain primitive haematopoietic cells retarded at an early stage of

haematopoietic differentiation and undergoing self-renewal rather than differentiation. It is more likely that these embryoid bodies contain cells that have not yet begun to undergo haematopoietic differentiation, as the self-renewal level is low.

5.3.4 Examination of gene expression during haematopoietic differentiation using semi-quantitative RT-PCR analysis

An examination of gene expression in these embryoid bodies could indicate when haematopoietic differentiation actually begins, and help discover at what developmental stage the mutation introduces a delay.

A time course of embryoid bodies was set up from CGR8 ES cells of the various DDS genotypes as previously described, then total RNA was extracted after 4, 8 and 12 days of differentiation. A CFU-A assay was also performed on embryoid bodies from the same time course (figure 5.5). The RNA was used to examine the expression patterns of genes which had previously been shown to alter their expression during embryoid body differentiation (Schmitt *et al.*, 1991; McClanahan *et al.*, 1993; Hole *et al.*, 1996). The genes chosen for analysis were adult *b1* and foetal *y* β-globin and G-CSF-R. The β-globin genes are believed to be very early indicators of the initiation of haematopoietic differentiation (J. Ansell, A. Cunningham, personal communication). Expression of the receptor for G-CSF has been suggested to be a marker for commitment of cells to myeloid lineages (McClanahan *et al.*, 1993). It was considered interesting to look for changes in expression patterns, attributable to the *Wt1* mutations, related to the observed delay

in haematopoiesis found using the CFU-A assay. These results are summarised in table 5.4 and illustrated in figure 5.11.

Previous studies have found the expression of foetal and adult β-globin to be an indicator of initiation of haematopoietic development. In a time course of wild-type embryoid bodies, expression of both types of β-globin are first detected by RT-PCR analysis at around days 3 to 5 of differentiation (Hole *et al.*, 1996). This is consistent with the results found here in CGR8 embryoid bodies, where adult β-globin is expressed at a high level at all the time points examined. Foetal β-globin expression is also seen at all time points in CGR8 wild-type EBs. In contrast, neither adult nor foetal β-globin expression is seen at the earliest time point in any of the mutant EBs. If β-globin expression is indeed a marker of the very earliest haematopoietic differentiation, this is consistent with the idea that the *Wt1* mutations present in these cells prevent the cells from entering the haematopoietic pathway for a time.

The G-CSF receptor is expressed mainly in precursor and mature neutrophils and myeloid cell lines (Park *et al.*, 1989). Performing a similar experiment with RT-PCR analysis of a time course of embryoid bodies, McClanahan *et al.* (1993) found significant induction of G-CSF-R during *in vitro* development, and suggested that this gene is a good candidate as a marker of myeloid lineage commitment. Schmitt *et al.* (1991) and Hole *et al.* (1996) also found its induction during an *in vitro* EB time course at days 12 and 8 of differentiation, respectively. As shown in figure 5.11 and table 5.4, CGR8 wild-type EBs show only a small increase in G-CSF-R expression from day 4 to day 8, followed by a large increase in expression between days 8 and

Table 5.4 Semi-quantitative RT-PCR results for CGR8 embryoid bodies and summary of CFU-A assay results for same embryoid body time course.

Genotype	CFU-A assay peak		Differentiation time (days)								
	Day	Height (%)	Adult globin			Foetal globin			G-CSF-R		
			4	8	12	4	8	12	4	8	12
+/+	6	20.6	+++	+++	+++	+	++	+++	+	+	+++
+/-	8	40.1	-	+	+	-	+	+	-	+	-
-/-	8	21.7	-	+	+	-	+	-	-	-	-

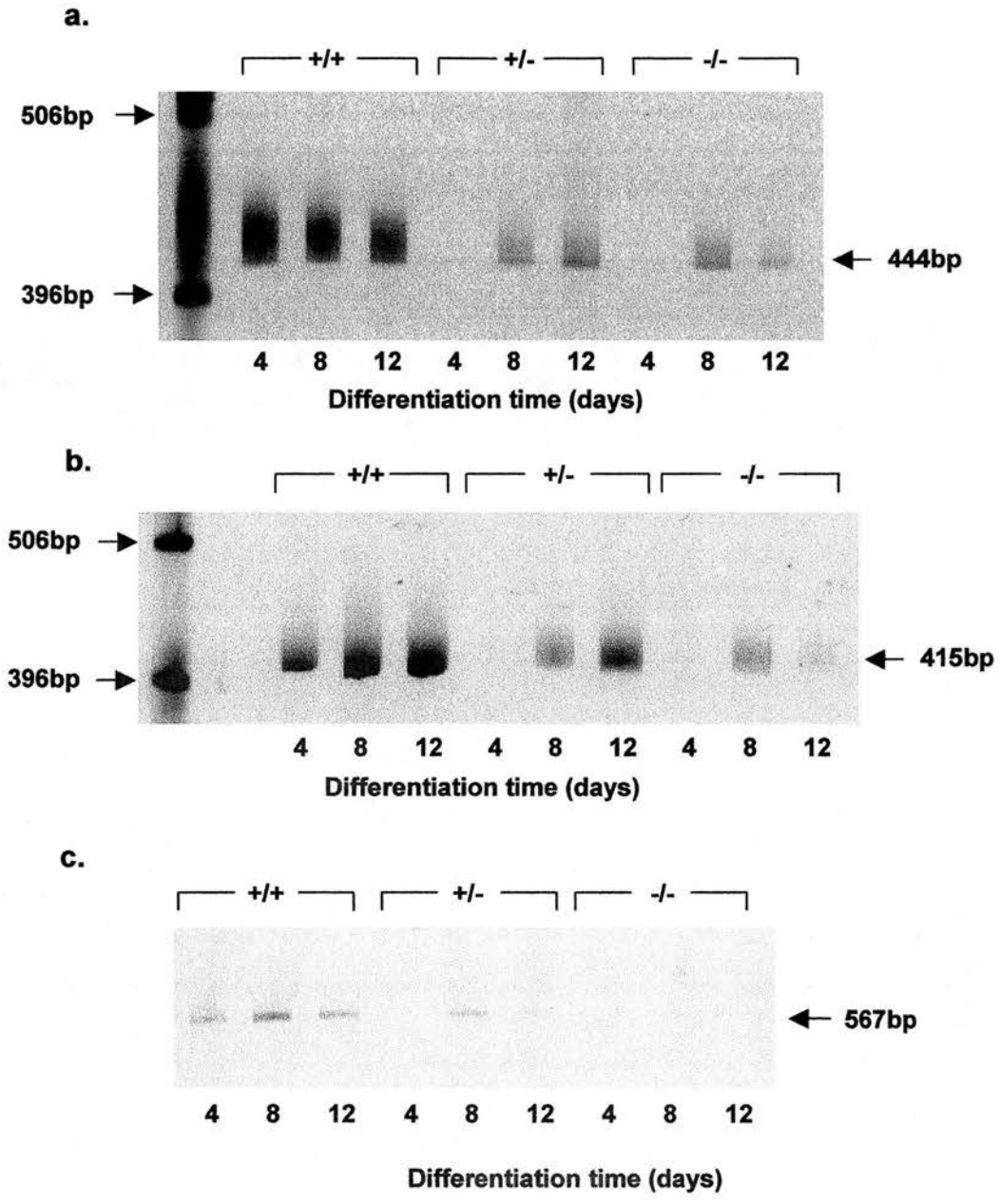


Figure 5.11 Autoradiographs of semi-quantitative RT-PCRs on cDNA from a CGR8 embryoid body time course

PCRs were performed on cDNA extracted from CGR8 embryoid bodies having undergone 4, 8 and 12 days of differentiation, taken from the same time course as those used for the CFU-A assay shown in figure 5.5.

a. Adult β-globin **b.** Foetal β-globin **c.** G-CSF receptor

12. Very little expression was seen in any of the mutant embryoid bodies, including no expression after 4 days of differentiation in any mutant. This lack of expression at day 4 would be as expected if a delay in haematopoietic induction is occurring in the mutant EBs. The very low level of G-CSF-R is surprising, though, if this is indeed a marker of myeloid commitment, as CFU-A positive colonies are believed to consist of mixed myeloid lineages, especially macrophages, and no difference in colony morphology has been observed between those produced from mutant and wild-type embryoid bodies. It could be that once the mutant embryoid bodies begin producing haematopoietic cells, these remain in a fairly primitive state until plated into the assay, as opposed to haematopoietic cells arising within wild-type EBs, which begin to mature before encountering the growth factors in the assay. Two lines of evidence refute this hypothesis, however. Firstly, the shape of the curve on a CFU-A graph indicates that CFU-A cells are produced within embryoid bodies, then their numbers begin to fall away as they mature into cells unable to produce a colony in the assay. This falling off of CFU-A numbers is detected in all mutant genotypes as well as wild-types, suggesting that the haematopoietic precursors in all EBs are maturing over time. Secondly, the evidence from scoring CFU-A assays at different times (see figure 5.8) revealed no difference in the rate of CFU-A production in mutant and wild-type cultures. If the mutant embryoid bodies contained a population of more primitive cells, a higher proportion of positive colonies would be expected to appear later, and this is not seen.

5.4 Discussion of chapter 5

It has been demonstrated here that cells predicted to contain no functional WT1 protein (ie not capable of normal DNA binding - Borel *et al.*, 1996; Pavletich and Pabo, 1991) are able to form embryoid bodies *in vitro*. These embryoid bodies are morphologically similar to those produced from wild-type ES cells and appear to contain a diverse range of cell types. Evidence that lineages derived from all three primitive germ layers are present in *Wt1* mutant embryoid bodies could be obtained by examining the expression of genes known to be markers for some of these lineages, using RT-PCR, Western analysis, or *in situ* hybridisation.

From the more specific studies of the effects of this mutation on haematopoietic development *in vitro*, some evidence has been obtained for a role for *Wt1* in this process. Embryoid bodies produced from cells with DDS-type truncations in either one or both *Wt1* alleles were found to take longer to differentiate into haematopoietic precursors than wild-type embryoid bodies. This was demonstrated using two independent methods for measuring haematopoietic differentiation: a direct stain for the presence of haem to reveal embryoid bodies in which erythropoiesis was occurring, and the CFU-A assay which indicates the presence of early haematopoietic precursors.

Examination of the self-renewal potential of the mutant and wild-type CFU-A colonies suggested that the *Wt1* mutation either prevents the cells from entering the haematopoietic differentiation process for a time or slows down the early stages of this process. Further evidence to support this came from looking at the expression of foetal and adult β-globin in these embryoid body time courses. Initiation of β-globin

expression is believed to occur at a very early stage of haematopoietic differentiation. This expression was seen to begin at a later time point in mutant compared with wild-type embryoid bodies. However, as the cells do go on to produce haematopoietic cells eventually, it is shown that the presence of wild-type WT1 is not an absolute requirement for haematopoietic differentiation to occur. WT1 could stimulate a step in the differentiation process, causing it to occur more rapidly in the presence of WT1 than without WT1. Alternatively, a completely separate pathway could be responsible for the haematopoietic differentiation seen in the *Wt1* mutant cells, either being activated later than the WT1-dependent pathway, or operating throughout, but at a slower rate than the WT1-dependent pathway.

This evidence points to a role for WT1 at the very beginning of haematopoietic differentiation. However, despite the occurrence of a phenotype due to lack of functional WT1 during *in vitro* ES cell differentiation, no evidence for haematopoietic defects has been reported in WT1 null mice (Kreidberg *et al.*, 1993). This may be because such defects have not yet been investigated, although the results from the CFU-A assays suggest that it could be worth doing. Homozygous *Wt1* null embryos die between day 13 and 15 of gestation (Kreidberg *et al.*, 1993), however, and such defects might not be apparent at this early stage. Also, a patient has been reported who is a constitutional homozygote for a missense *WT1* mutation (Kikuchi *et al.*, 1995), yet has no apparent haematopoietic defects. According to the results of the *in vitro* CFU-A assay, despite a delay, haematopoietic development can proceed in the absence of functional WT1, which may happen *in vivo* soon enough to prevent disruption of haematopoietic development. This does not mean, however, that subtle differences in the haematopoietic system do not result from lack of WT1. The patient

reported by Kikuchi *et al.* (1995) was only 13 months old, and could perhaps have gone on to develop leukaemia in later life. Also, in neither the Kikuchi *et al.* study or the Kreidberg *et al.* (1993) null mouse paper was any detailed examination made of the haematopoietic system. This therefore does not rule out the possibility that subtle differences, perhaps in the proportions of the different blood cell types, are present.

The results from the DDS heterozygous cells, which also showed a delay relative to wild-type cells, are interesting, as the mutations found in AML cases have been reported to resemble the mutation in the heterozygous cells, in that they are usually found in a heterozygous state and lead to the disruption of the WT1 zinc finger region (King-Underwood *et al.*, 1996; King-Underwood and Pritchard-Jones, 1998). This is evidence for the idea that the observed delay in haematopoiesis caused by the presence of *Wt1* mutations may be directly related to the mechanism by which the heterozygous mutations found in AML lead to leukaemia.

How could a mutation that delays entry into haematopoiesis be involved in leukaemogenesis? As the haematopoietic stem cells (HSCs) are produced during prenatal development, the mutation would have to occur at this stage. It is possible that a halt in the differentiation process of a cell, caused by a mutation like this, could increase the chance of some other genetic event occurring. In other words, it could make a 'second hit' in the same cell, or its progeny, more likely, perhaps in a gene required for the regulation of proliferation. This cell could then become one of the many quiescent HSCs which are found in a mature mammal. Only a small proportion of the HSCs are active at any one time, and these few are responsible for the maintenance of the cell population of the haematopoietic system (Lajtha, 1979). If the mutant HSC became activated, as part of the normal haematopoietic

maintenance processes, it could then begin to proliferate out of control, as a regulatory mechanism for this process would be non-functional.

Of course, it is ectopic expression of WT1 that is the predominant observation in human leukaemias, so possibly examination of haematopoietic differentiation in cells lacking functional WT1 is unlikely to elucidate matters. It could be that this finding in leukaemias represents a separate, later, time point at which WT1 acts, which is related to the observed down-regulation of WT1 during early haematopoietic differentiation (Sekiya *et al.*, 1994; Phelan *et al.*, 1994). Perhaps an examination of the performance in this assay of ES cells overexpressing *Wt1* would give a better indication of the role of WT1 in leukaemogenesis. Although the presence of *WT1* mutations (King-Underwood and Pritchard-Jones, 1998) in leukaemias, possibly producing protein with reduced or altered WT1 function, and the identification of leukaemias with overexpression of apparently wild-type WT1 (Miwa *et al.*, 1992; Inoue *et al.*, 1994) appears contradictory, it is possible that two different routes to leukaemia can be achieved by opposite effects on the same gene. There is evidence to suggest that WT1 overexpression or inappropriate expression will maintain cells in, or return them to, a proliferative state, despite the presence of differentiation-inducing stimuli (Svedberg *et al.*, 1998; Inoue *et al.*, 1998). It is therefore easy to see how ectopic WT1 expression could cause leukaemia. As has been demonstrated here, *Wt1* mutations which affect the zinc finger region of the protein cause a delay in haematopoietic differentiation from ES cells. This delay could increase the chance of a cancer-causing mutation occurring in a haematopoietic precursor, resulting in leukaemia later in life when the resulting HSC becomes activated.

It should be pointed out that the *WT1* mutations detected in leukaemias have been observed in a heterozygous form, leading to truncated proteins such as the ones used here (King-Underwood and Pritchard-Jones, 1998). The embryoid bodies produced from such heterozygous cells did reveal a delay in haematopoiesis similar to that seen in the compound heterozygotes and homozygotes. These two results would indicate that the role of WT1 in haematopoiesis could be a) sensitive to dosage of the wild-type protein, b) the mutant forms are somehow affecting the wild-type, or c) that the mutant forms have a novel, dominant function. The second suggestion is unlikely, as there is no wild-type protein in the compound heterozygotes or homozygotes, which show the same effect. The extremely low level of mutant protein present in these genotypes also makes the last hypothesis doubtful, although as some mutant protein is present, it could be possible.

The CFU-A assay has shown that a DDS-type mutation in only one *Wt1* allele is sufficient to cause a delay in haematopoiesis, yet DDS patients are constitutional heterozygotes for this type of mutation, and leukaemia has not been reported as a common secondary malignancy in DDS, nor are defects of the haematopoietic system a feature of DDS. However, subtle haematopoietic abnormalities have not been investigated.

It could be that *Wt1* expression in early haematopoietic precursors is important not only to maintain the cells in a proliferative state, but also fulfils an important role in the maturation process, enabling further haematopoietic differentiation to then occur. WT1, in its role as a transcription factor, may trigger the expression of certain genes required for further differentiation along the haematopoietic pathway. In *Wt1* mutants, expression of these genes is not induced,

so differentiation cannot proceed. However, haematopoiesis does eventually occur, and no defects of the haematopoietic system have been reported in *Wt1* null mice, so transcription of these genes must eventually occur via some other mechanism. The results reported here, however, suggest that it could be worthwhile to make a detailed examination of the haematopoietic system in *Wt1* null embryos and heterozygous null mice. It is possible that the haematopoietic microenvironment within the embryoid bodies alters at some point in the differentiation process and begins to produce something which overrides the requirement for WT1 and triggers an alternative pathway.

6. Use of cDNA expression arrays to investigate gene expression in Wt1 mutant and wild-type ES cells

As already discussed in section 1.5.2, many genes have been suggested as transcriptional targets of WT1. A list of these is found in table 1.1. Such genes were linked with WT1 either because their expression was found to alter in Wilms' tumours compared with normal kidney, or because the suggested WT1 consensus sequence was found in their promoter regions. *In vitro* experiments using promoter constructs provided further evidence for these genes being direct WT1 targets (Drummond *et al.*, 1992; Gashler *et al.*, 1992). However, more recent work suggests that at least some of these results may be artifacts, as the examination of endogenous target gene expression rather than constructs found no evidence for an effect of WT1 on some of these genes (Englert *et al.*, 1995a). These experiments still used transfection, often transient, of WT1 cDNA into the cell, meaning that only one of a possible 16 WT1 isoforms was represented. This obviously does not reflect the normal situation either *in vivo* or *in vitro*, so the production of ES cells with *Wt1* mutations has provided a useful tool for the examination of endogenous target gene expression in cells where the expressed WT1 is also endogenous and under the control of its own promoter. In an attempt to identify genes that are genuinely under the transcriptional control of WT1, cDNA from RA-differentiated ES cell progeny of wild-type cells and cells harbouring *Wt1* mutations in the zinc finger domain was used to probe cDNA arrays fixed on membranes. Macroarray technology allows the expression of many, in this case 588, genes to be studied simultaneously. The arrays

used have cDNA spots arranged into functional groups on a nylon membrane (figure 6.1), and the full list of genes represented can be found at:

http://www.clontech.com/archive/JAN98UPD/Atlaslist.html

A cDNA probe was made from polyA$^+$ RNA, with the incorporation of dNTPs containing ^{32}P-dATP to radioactively label the transcripts. The resulting labelled, mixed, cDNA population was used to probe the Mouse Atlas™ expression array (Clontech). Hybridisation occurred between species represented in the cDNA probe and cDNA spots fixed to the membrane, giving a signal on an autoradiograph, or using a phosphorimager, the intensity of the signal produced being proportional to the abundance of the specific cDNA in the probe (Kelly and Rizzino, 2000). The resulting pattern of signals from two different genotypes or from differentiated and undifferentiated cells of the same genotype could be compared, revealing the expression levels of the various genes represented on the membrane. The results were analysed using Clontech's AtlasImage™ software, which compares the intensities of each spot on the membrane between two different membranes.

6.1 Comparison of gene expression in retinoic acid-differentiated and undifferentiated wild-type ES cells

The comparison between undifferentiated wild-type CGR8 ES cells and their RA-differentiated progeny was performed to provide information about some of the cell lineages that are produced when ES cells are induced to differentiate using all-trans retinoic acid (figure 6.2). This continues the work described in section 4.3.4,

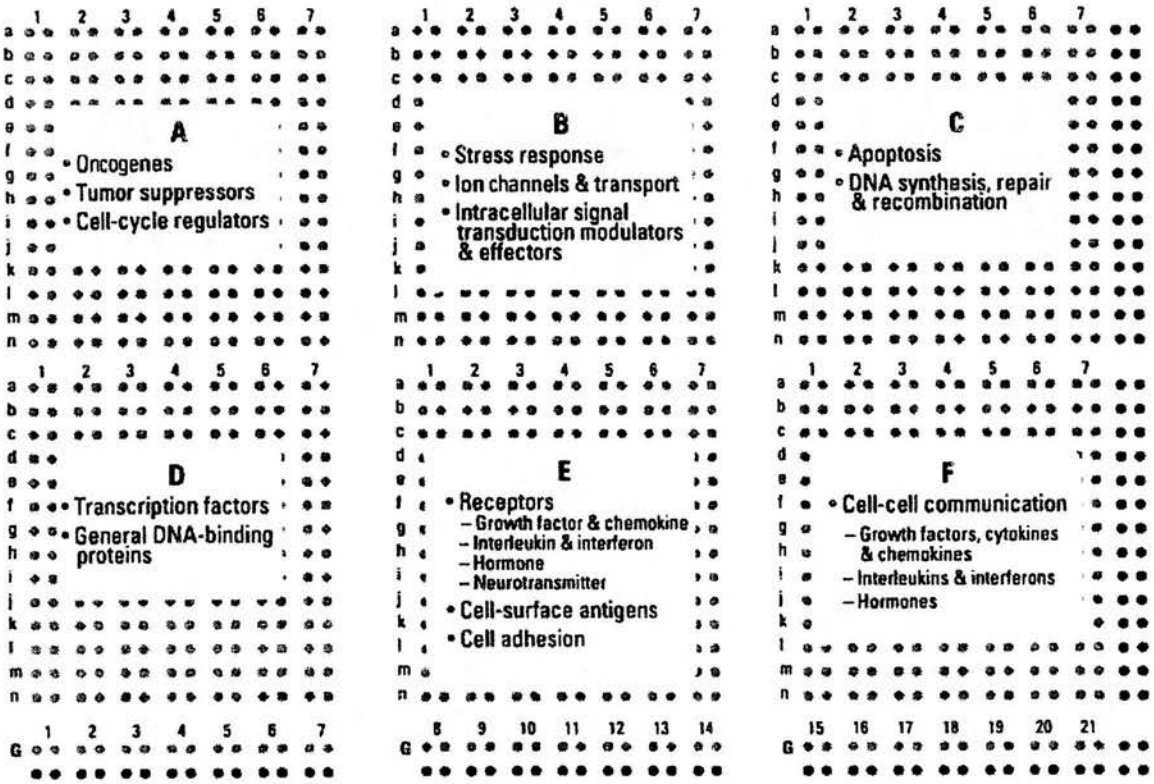
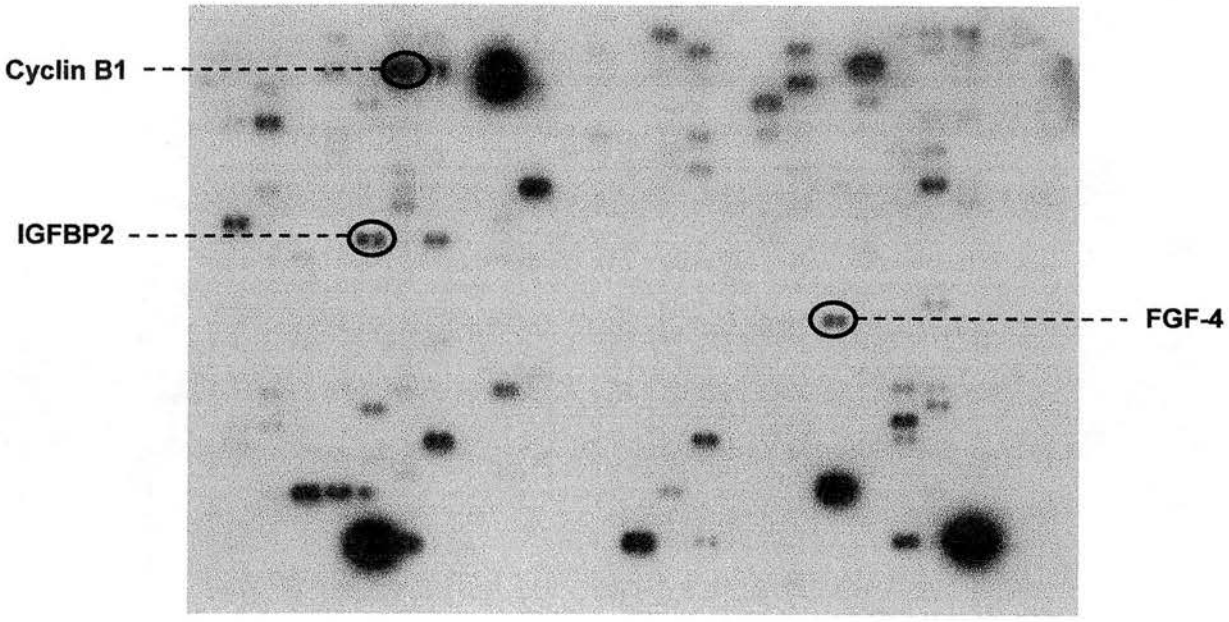
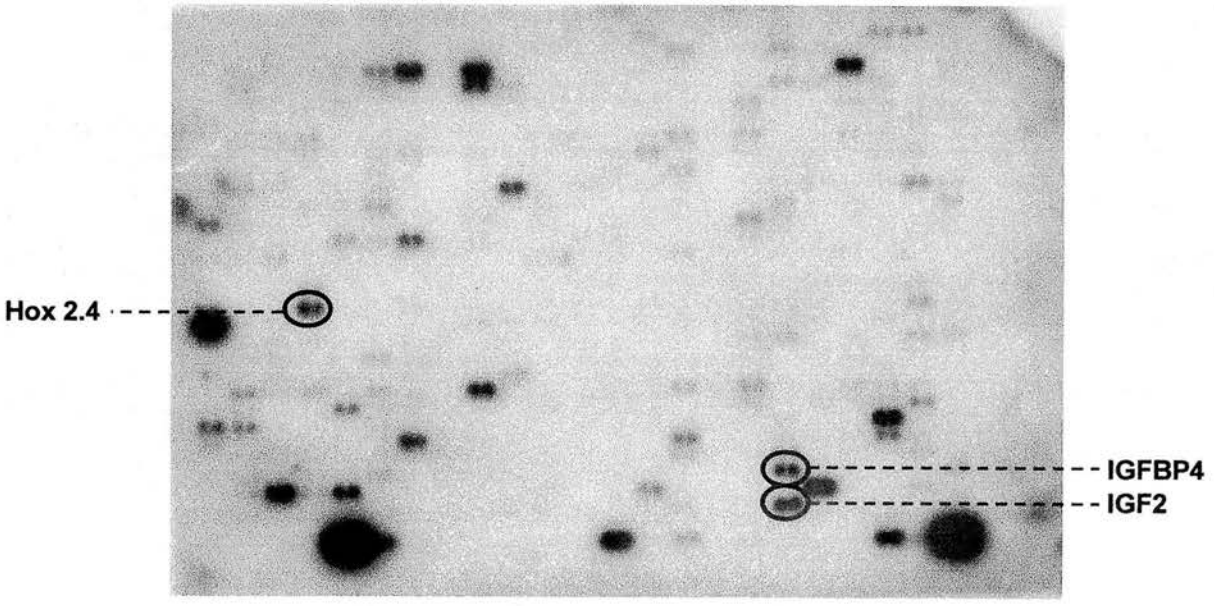


Figure 6.1 Layout of the Clontech Atlas™ mouse version I expression array

Regions A-F contain 200-500bp cDNA fragments placed into functional groupings. Region G (at the bottom of the array) consists of negative control plasmid and bacteriophage cDNAs, plus nine housekeeping control cDNAs which are used to normalise mRNA abundance. The darker spots at the very bottom and far right of the array are genomic DNA, which are intended for use in orientation of the hybridised array, enabling identification of the coordinates of signals of interest.



a.



b.

Figure 6.2 Comparison of gene expression in CGR8 wild-type retinoic acid-differentiated and undifferentiated ES cells using mouse Atlas expression arrays.

a. Undifferentiated wild-type

b. Differentiated wild-type

Circles indicate the positions of some differentially expressed genes.

where it was shown that the majority of cells express WT1 protein after 96 hours of 5μM RA-differentiation. It was hoped that the genes found to be expressed in the differentiated cells would include some markers of cell lineage, enabling an impression to be obtained of the cell types present in a differentiated cell population.

The results from this experiment were not compared using the AtlasImage™ software, but by visual assessment, so they are not quantitative. Genes were identified whose expression was seen to be substantially higher on one membrane than the other, relative to the other signals present on each membrane. The results are displayed in table 6.1.

Table 6.1 Genes that were either substantially upregulated or downregulated after four days of RA differentiation of wild-type CGR8 ES cells

Higher in undifferentiated cells	**Higher in differentiated cells**
B-myb proto-oncogene	Tight junction protein ZO-1
p53 tumour suppressor	Protein kinase C theta
c-kit proto-oncogene	Zyxin
Cyclin B1 (G2/M specific)	Plasma glutathione peroxidase
IGFBP2	*TDAG51*
Heat shock protein 84 (*Hsp90β*)	Pre-adipocyte factor 1 (*Pref-1*)
Heat shock protein 86 (*Hsp90α*)	Homeobox protein 2.1 (*Hoxb5*)
BAG-1 BCL-2 binding protein	Homeobox protein 2.4 (*Hoxb8*)
Glutathione S-transferase mu 1	*IGFBP-4*
Glutathione S-transferase pi 1	Insulin-like growth factor 2 (*Igf2*)
MSH2 DNA mismatch repair protein	Butyrate response factor 1
Lung Kruppel-like factor (*LKLF*)	Serine protease inhibitor homologue J6
GTP-binding protein *Cdc42*	
Fibroblast growth factor 4 (*FGF-4*)	
Laminin receptor 1	

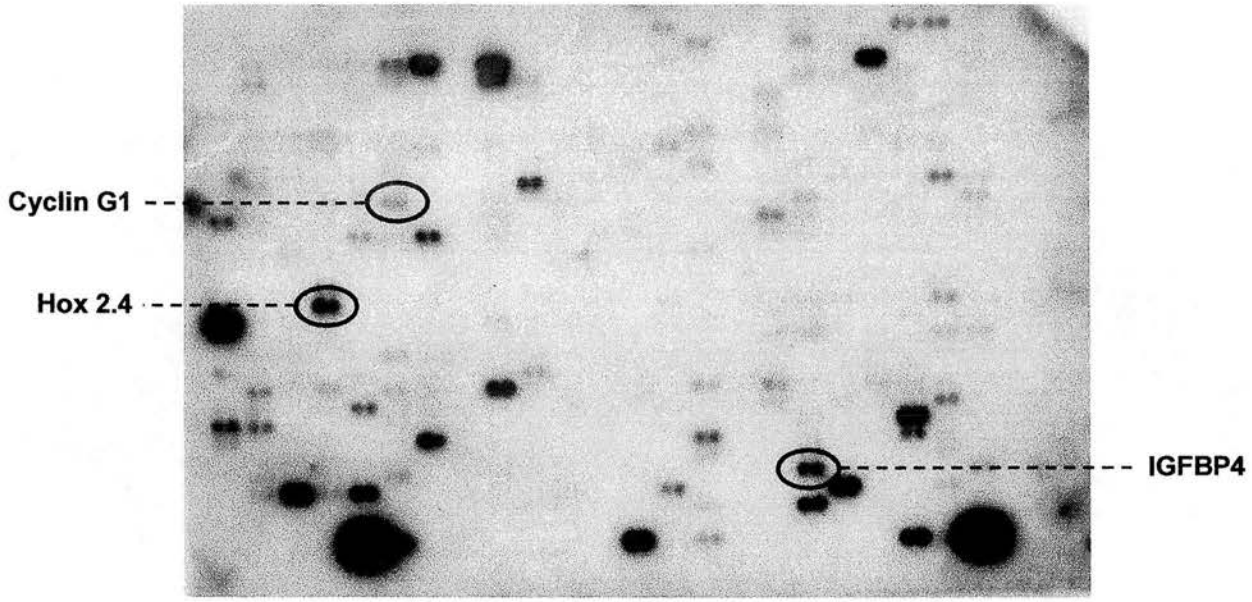
Although it had already been determined from the evidence provided by Northern, Western and immunofluorescence analysis that *Wt1* expression occurs in the differentiated cells at a higher level than it does in the undifferentiated cells

(section 4.3.4), this expression was not high enough even in the differentiated cells to be detected by this method.

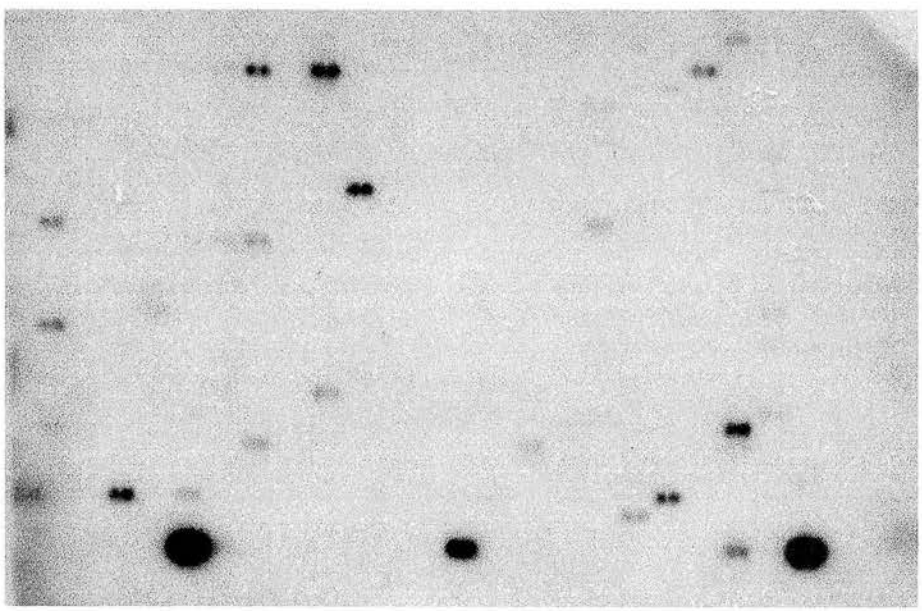
6.2 Comparison of gene expression in retinoic acid-differentiated wild-type and DDS compound heterozygous CGR8 and wild-type and DDS homozygous E14 ES cell progeny

Comparisons between gene expression in CGR8 wild-type and DDS compound heterozygous (figure 6.3) and between E14 wild-type and DDS homozygous (figure 6.4) differentiated ES cell progeny were made using Clontech mouse Atlas™ macro-arrays. The cells had first been induced to differentiate for 96 hours using 5μM all-trans retinoic acid (section 3.1.8), as it was necessary to achieve WT1 expression in the cells before making comparisons between the different genotypes. These results were then compared to identify differences that were consistent between the two cell lines, indicating effects attributable to *Wt1* mutation, rather than genetic changes elsewhere in the genome.

Results were analysed using AtlasImage™ software, which normalises spot intensities relative to other spots on the same membrane, including those for house-keeping genes, before making comparisons between membranes, to account for differences in overall hybridisation efficiency and probe labelling. The difference between the normalised intensities was determined, as was the ratio of the normalised intensities of the mutant to wild-type spots. Threshold values for the ratio



a.



b.

Figure 6.3 Comparison of gene expression in CGR8 wild-type and DDS compound heterozygous (*Wt1*^{tmT396}/*Wt1*^{tmT403}) retinoic acid-differentiated ES cells using mouse Atlas expression arrays.

a. Differentiated wild-type

b. Differentiated DDS compound heterozygote

Some of the more obvious differences are indicated by circles.

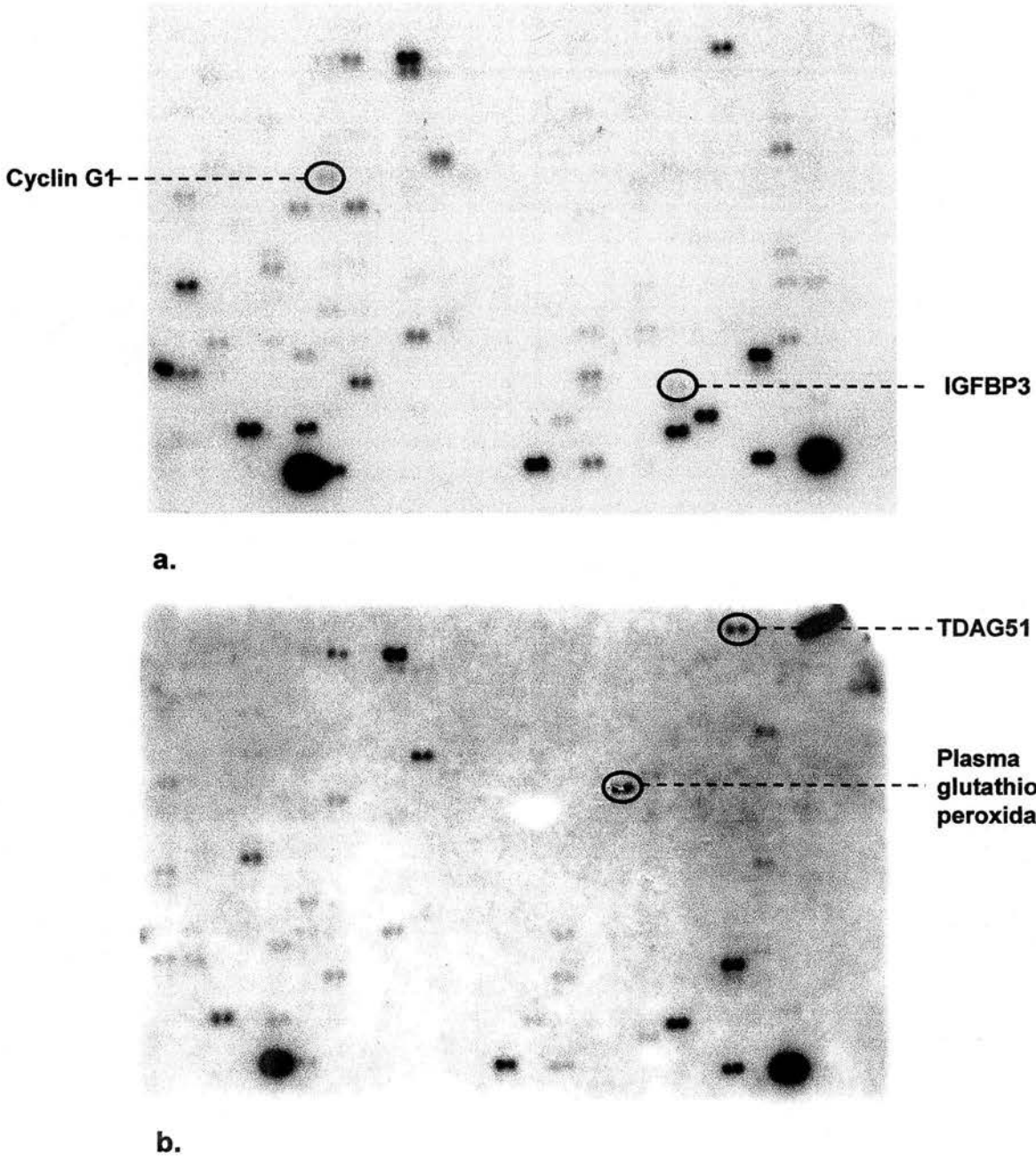


Figure 6.4 Comparison of gene expression in E14 wild-type and high G418 DDS homozygous ($Wt1^{tmT396}/Wt1^{tmT396}$) retinoic acid-differentiated ES cells using mouse Atlas expression arrays.

a. Differentiated wild-type

b. Differentiated DDS homozygote

Circles indicate the positions of some differentially-expressed genes.

between the mutant and wild-type intensities outside which the value was considered to be significant were determined by the software from the data, as were actual values for this difference above which the results were considered to be significant. Spots were considered to be significantly different from one another if the difference exceeded a certain value and if the ratio fell outside the range 0.59-1.69. To be considered significant, for the CGR8 comparison, the difference had to exceed 9335 normalised intensity units and for the E14 comparison, the difference had to exceed 225 normalised intensity units. Table 6.2 shows the genes that were considered to be significantly different for at least one of the cell lines, and for which the results from the other cell line are also significant, or nearly so.

Table 6.2 Genes showing altered expression relative to wild-type cells in both CGR8 DDS compound heterozygous and E14 DDS homozygous cells

Gene	CGR8		E14	
	Diff.	Ratio	Diff.	Ratio
G_2/M specific cyclin (*cyclin G1*)	-13034	0.43	-207	0.47
Plasma glutathione peroxidase precursor	23910	2.04	778	6.85
RAD23 UV excision repair protein B	-11932	0.54	-308	0.42
Insulin-like growth factor binding protein 3	-12975	0.15	-240	<0.15
Insulin-like growth factor binding protein 4	-35966	0.21	-299	<0.15
Heat shock 86 kDa protein (*Hsp86*)	-22349	0.48	-283	0.55
T-cell death-associated protein (*Tdag51*)	14193	1.68	620	8.75
RAD21 DNA repair protein	8136	1.98	335	3.38
14-3-3 protein zeta/delta	-15357	0.69	-476	0.58

Diff. – difference between normalised intensities of the two spots.

Cyclin G1, *RAD21* and 14-3-3 protein zeta/delta are included in the table because the results are significant for one cell line, and very near to the thresholds for significance in the other.

Several genes have been suggested to be transcriptional targets of the WT1 protein (section 1.5.2). As some of these are present on the expression arrays, it was decided to examine the results obtained for these in the comparisons between wild-type cells and those with two *Wt1* mutations. The results are presented in table 6.3. The values of difference in spot intensity and ratios corresponding to significant changes in gene expression are the same as for table 6.2.

Table 6.3 Expression of putative WT1 target genes relative to wild-type cells in CGR8 DDS compound heterozygous and E14 DDS homozygous cells

Gene	CGR8		E14	
	Diff.	Ratio	Diff.	Ratio
Early growth response protein 1 (*Egr1*)	-16315	0.40	228	2.98
Insulin-like growth factor 2 (*Igf2*)	708	1.01	-1312	0.15
Insulin-like growth factor 1 receptor alpha	-1568	<0.21	118	6.13
Platelet-derived growth factor A chain	-956	<0.21	148	9.22
Transforming growth factor beta 1 (*Tgf-β1*)	-2622	<0.21	13	1.87
Colony-stimulating factor 1 (*Csf-1*)	-1886	<0.21	89	6.93
c-myb proto-oncogene	-2041	0.7	28	6.6
Inhibin α	-3398	0.26	-28	0.15
Ornithine decarboxylase	-720	0.96	-280	0.58
c-myc proto-oncogene	-6022	0.48	-26	0.73
B-cell lymphoma protein 2 (*bcl-2*)	-3816	<0.21	27	3.38
Insulin receptor (*IR*)	-940	<0.21	40	6.00
Cyclin-dependent kinase inhibitor 1 (*p21*)	7135	6.4	26	2.04

Expression of none of these genes was significantly affected in the same direction by the presence of the *Wt1* mutations in both CGR8 and E14 cells. *Egr1* was significantly altered in both cell lines, but expression is much lower in the mutant than the wild-type in the CGR8 cell line and higher in the mutant than the wild-type in the E14 line. Ornithine decarboxylase and *Igf2* are significantly altered

in the E14 mutant compared to the wild-type, but as this difference is not also found in the CGR8 cells, it cannot be attributed to the *Wt1* mutation.

6.3 Comparison of gene expression in retinoic acid-differentiated wild-type and DDS heterozygous CGR8 ES cells

A comparison between gene expression in wild-type and DDS heterozygous ES cells was only made for the CGR8 line (figure 6.5). This means that any results which differ from those obtained with the compound heterozygotes and homozygotes may be due to unknown genetic changes which have occurred in this clone, and may be unrelated to the *Wt1* mutation present. This is particularly true as the heterozygous cells used here are from clone 10, while the compound heterozygous CGR8 clone used was derived from heterozygous clone 15.

It was considered important to look at gene expression in the DDS heterozygous cells, as it is in a heterozygous form that DDS mutations occur in affected individuals, and because this mutation has already been demonstrated to produce a phenotype *in vivo*. Patek *et al.* (1999) found that this same mutation induced symptoms of DDS in chimaeric and heterozygous mice, so any changes in gene expression levels which are responsible for this pathology may be detectable *in vitro* using this method.

Regarding the heterozygous cells, the data can be divided into three categories: (1) Genes whose expression altered in both the heterozygotes and compound heterozygotes/homozygotes (table 6.4a); (2) Genes whose expression altered in the

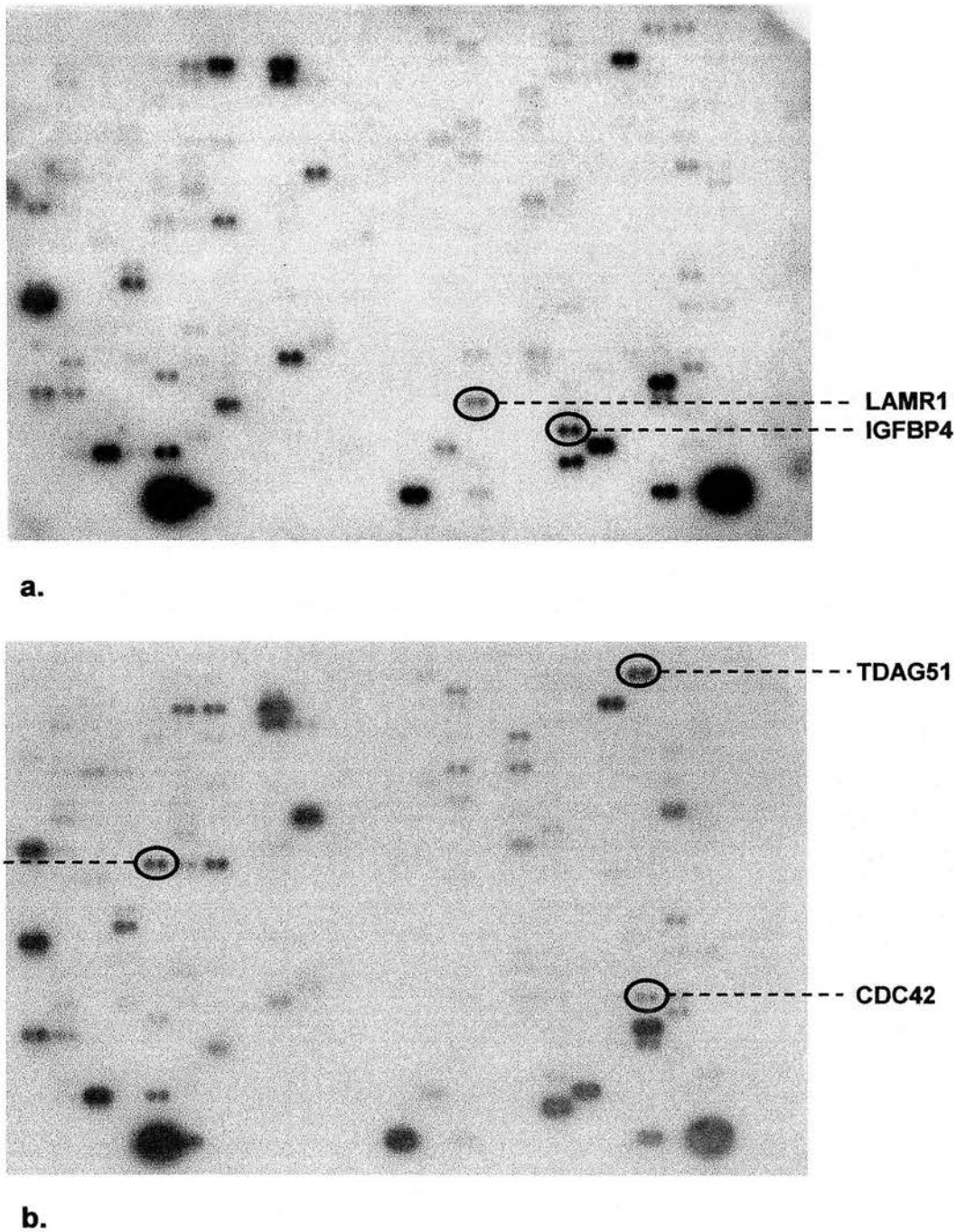


Figure 6.5 Comparison of gene expression in CGR8 wild-type and DDS heterozygous (*Wt1/Wt1*^{tmT396}) retinoic acid-differentiated ES cells using mouse Atlas expression arrays.

a. Differentiated wild-type

b. Differentiated DDS heterozygote

Circles indicate the positions of some differentially-expressed genes.

compound heterozygotes/homozygotes but not the heterozygotes (table 6.4b) and (3) Genes whose expression altered in the heterozygotes only (table 6.4c). This last group of results is that most likely to have been affected by changes specific to the one heterozygous clone. To be significant in this comparison, the ratio between the two intensities must lie outside the range 0.59-1.69, and the difference between the two intensities must exceed 10,069.

Table 6.4 Comparison of gene expression in CGR8 wild-type and DDS heterozygous RA-differentiated ES cell progeny

a. Genes showing altered expression relative to that in wild-type cells in DDS heterozygotes as well as DDS compound heterozygotes/ homozygotes

Gene	**Diff.**	**Ratio**
Insulin-like growth factor binding protein 3	-15344	<0.17
Insulin-like growth factor binding protein 4	-30070	0.34
T cell death-associated protein (*Tdag51*)	30572	2.47

b. Genes showing altered expression relative to wild-type cells in DDS compound heterozygous/homozygous cells, but not DDS heterozygous cells

Gene	Diff.	Ratio
Plasma glutathione peroxidase precursor	2784	1.12
RAD23 UV excision repair protein B	5976	1.23
Heat shock 86kDa protein (*Hsp86*)	8803	1.20
G2/M specific cyclin (*cyclin G1*)	-6095	0.73
14-3-3 protein zeta/delta	-8750	0.82

c. Genes showing altered expression relative to that in wild-type cells in DDS heterozygous cells, but not DDS compound heterozygous/ homozygous cells (showing comparison with results for CGR8 compound heterozygote)

Gene	+/-		-/-	
	Diff.	Ratio	Diff.	Ratio
Insulin-like growth factor binding protein 2	23309	2.00	2574	1.11
Golgi 4-transmembrane spanning transporter	15354	2.18	-631	0.95
BAX membrane isoform alpha	22943	3.49	2909	1.32
Laminin receptor 1 (*LAMR1*)	-30828	<0.17	2821	1.09
CDC42 GTP-binding protein	19535	3.67	-1508	0.79
Cathepsin B1 (*CTSB*)	13728	3.24	-4369	0.28

+/- CGR8 heterozygous ($Wt1/Wt1^{Tmt396}$) cells

-/- CGR8 compound heterozygous ($Wt1^{Tmt396}/Wt1^{Tmt403}$) cells

An interesting point to note here is that there are approximately the same numbers of genes in the mutant cells showing increased as decreased expression in the first two parts of the table. In the third part, which contains genes whose expression altered in the heterozygous but not compound heterozygous/homozygous cells, most of the genes showed increased expression in the mutant, suggesting that this mutation in a heterozygous form either prevents a repressive effect of the WT1 protein or produces a novel activating effect. This will be discussed in section 6.5.

6.4 Confirmation of expression array results using RT-PCR analysis

Some of the genes identified by the Atlas™ expression arrays as being differentially expressed between *Wt1* mutant and wild-type ES cells were examined further using RT-PCR to confirm the results. The genes chosen were some of those that showed consistent differences between double mutant and wild-type in both CGR8 and E14 cells. Primer pairs, each of which spanned an intron, were designed for *cyclin G1*, *Hsp86* and *IGFBP-4* so that any contaminating genomic DNA sequences would probably not be amplified due to their size, and would produce a PCR product of a larger size even if amplification did occur. Despite not knowing the actual cDNA sequences bound to the Atlas™ membranes, it is certain that at least part of the sequence amplified here was not represented on the membrane, as the expected PCR products were all in the region of 800bp, whereas those bound to the membrane were 200-500bp. This means that this PCR amplification will include

a different part of the gene of interest, and so constitutes an independent method that can therefore be used to confirm the macroarray results.

Cyclin G1 was thought to be an interesting gene for study as it is required for the cell cycle transition from G_2 to M, a transition at which WT1 is also believed to be important (Yamagami *et al.*, 1996). WT1 is already known to interact with HSP70, leading to transcriptional upregulation of the *Hsp70* gene (Maheswaran *et al.*, 1998). Unfortunately this gene is not represented on the expression array, but a related gene, *Hsp86*, was found to be expressed at lower levels in the DDS compound heterozygous and homozygous cells, so expression levels of this gene were confirmed by RT-PCR. *IGFBP-4* was also examined, as it had previously been suggested as a familial Wilms' tumour gene (Rahman *et al.*, 1996).

Total RNA was extracted from RA-differentiated wild-type, heterozygous and compound heterozygous/homozygous CGR8 and E14 ES cells. This was treated with DNaseI to remove any genomic DNA contamination, then underwent first strand cDNA synthesis. RT-PCR was performed on this cDNA, and also control samples made with no reverse transcriptase (no RT), using primers for *cyclin G1*, *Hsp86* and *IGFBP-4*, with the number of PCR cycles chosen to ensure that the intensity of the band produced was proportional to the amount of template. A glyceraldehyde phosphate dehydrogenase (*GAPDH*) PCR was used as a measure for the cDNA concentration, to standardise the other PCR results (figure 6.6). PCR products of the expected size only were obtained in each reaction, and no product was obtained in any no RT control. PCR primers, conditions and band sizes can be found in appendix II.

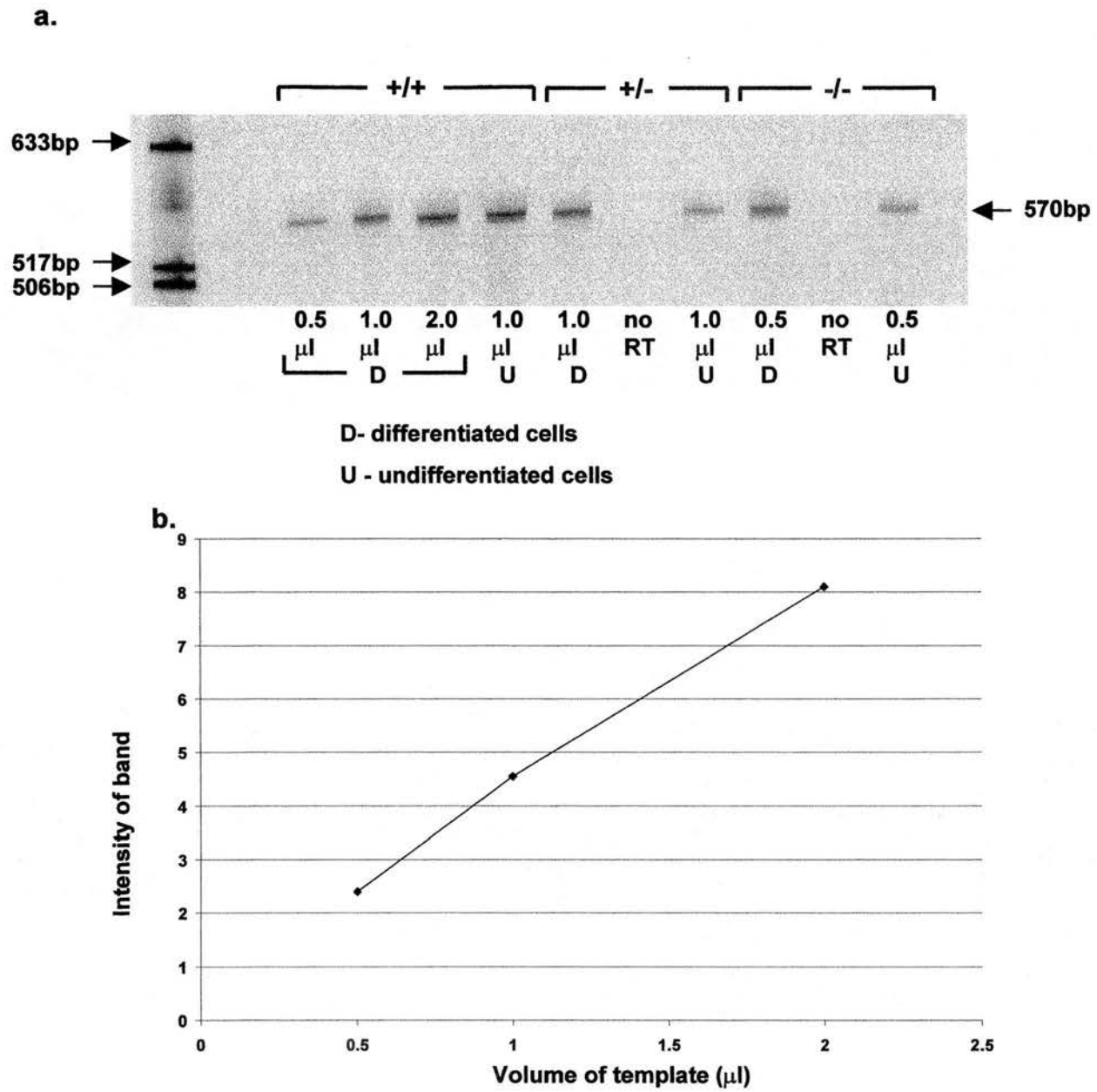


Figure 6.6 *GAPDH* RT-PCR used to standardise amount of template for differentially-expressed gene RT-PCRs

a. Autoradiograph of *GAPDH* RT-PCR, showing intensities of bands produced.

b. Standard curve to show that after 16 cycles of PCR, intensity of signal produced is proportional to amount of template added.

IGFBP4 was seen to be far more highly expressed in the differentiated than undifferentiated cells (figure 6.7), which agrees with the result from this comparison performed on wild-type cells (section 6.1). The RT-PCR analysis shows expression to be low in undifferentiated cells within the other genotypes as well, which would be expected, as these cells express WT1 at an extremely low level, so genotype should have no effect. In the differentiated cells, *IGFBP-4* expression is approximately twice as high in the wild-type cells as in the *Wt1* mutant cells. This difference is slightly lower than predicted from the expression array results, as the ratios between wild-type and mutant expression levels for the DDS heterozygote and DDS compound heterozygote were 0.34 and 0.21, respectively. However, this analysis was performed on different clones than those used in the expression array analysis, so some variability could be expected, and the levels in the mutant cells are substantially lower than in the wild-type, thus confirming the result from the expression arrays.

Cyclin G1 expression is detected at a much higher level in the differentiated than undifferentiated wild-type cells by RT-PCR (figure 6.8), although this was not seen on the expression array results (section 6.1). As the comparison between differentiated and undifferentiated images was only by visual assessment, only the most obvious differences between the two membranes were regarded as being real results, so a less dramatic difference in *cyclin G1* levels would have been discounted. Again, the fact that different clones were used for the expression arrays as for RT-PCR could partly explain the difference. The variation in *cyclin G1* levels in undifferentiated cells between the different DDS genotypes was not examined by

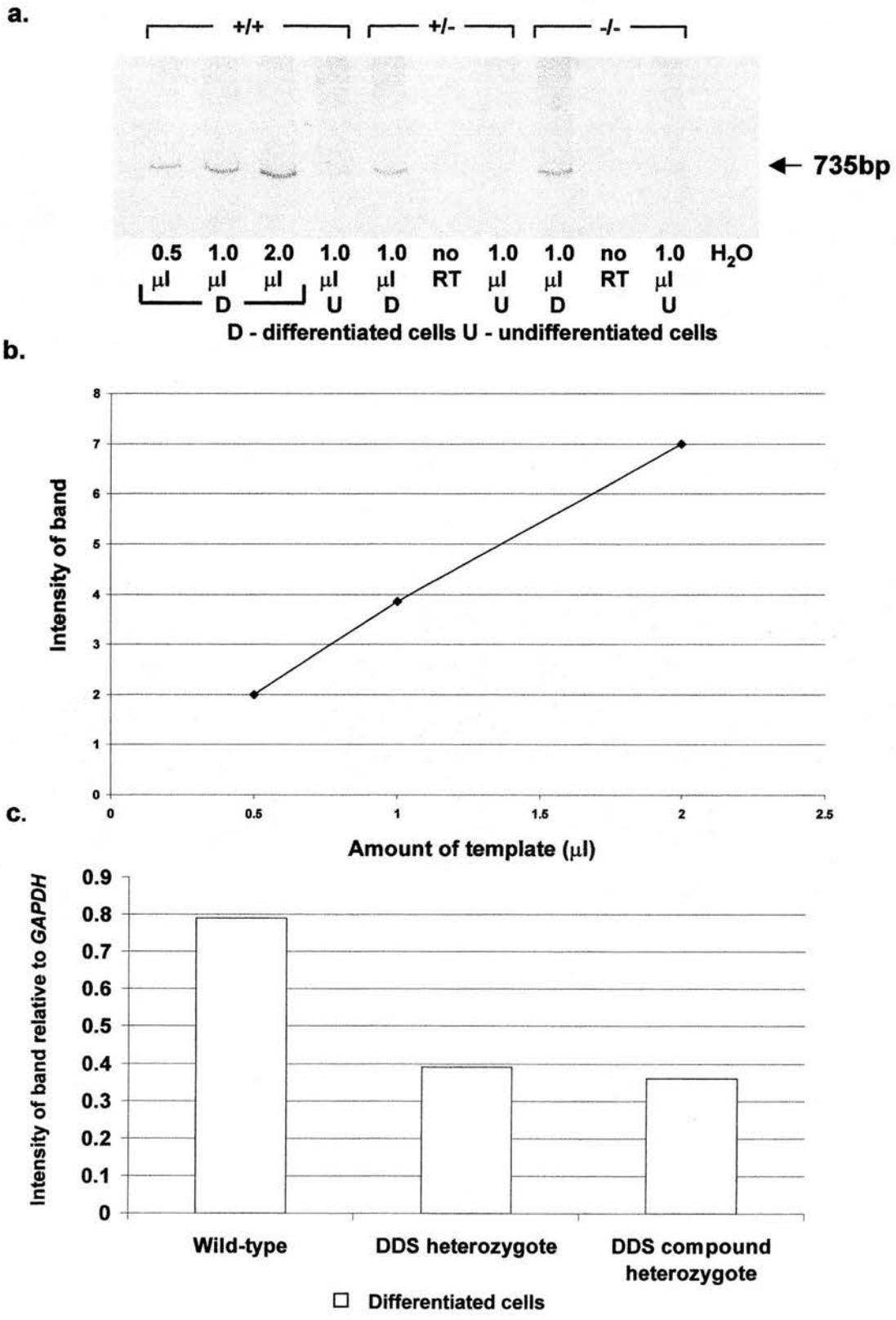
Legend to figure 6.7:

a. Autoradiograph of quantitative RT-PCR, showing intensity of bands produce with *IGFBP-4* primers.

b. Standard curve to show that after 16 cycles of PCR, intensity of band produced proportional to amount of template added.

c. Results corrected for amount of template using *GAPDH* PCR results. The ban intensities produced by all the undifferentiated cells fell below the range o template concentrations for which linearity had been established, so could not b estimated accurately.

Figure 6.7 Comparison of IGFBP-4 expression in CGR8 mutant and wild-type ES cells using cDNA expression arrays and RT-PCR analysis.



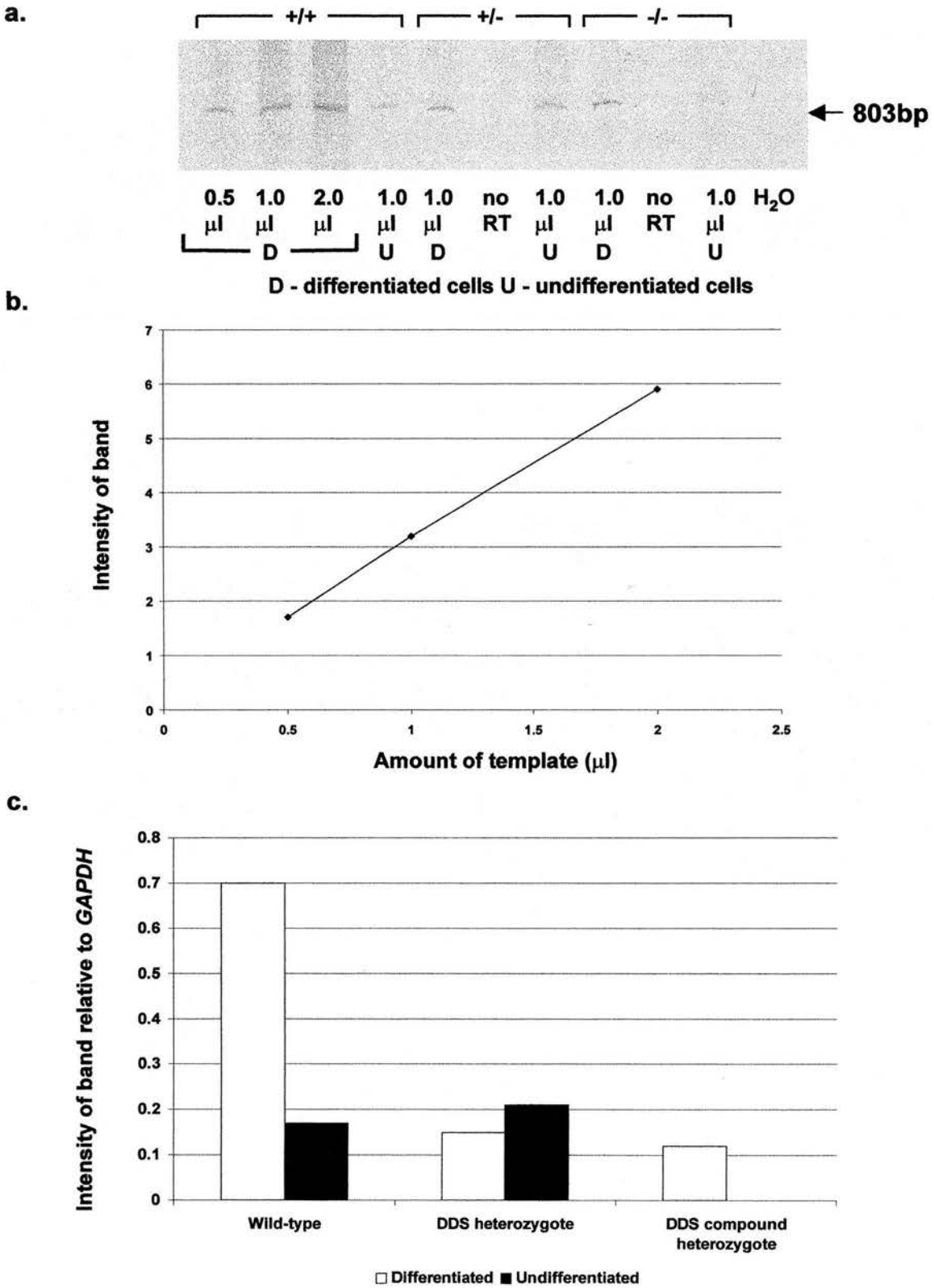
Legend to figure 6.8:

a. Autoradiograph of quantitative RT-PCR, showing intensity of bands produced with *cyclin G1* primers.

b. Standard curve to show that after 12 cycles of PCR, intensity of band produced is proportional to amount of template added.

c. Results corrected for amount of template using *GAPDH* PCR results. The band intensity produced by the undifferentiated compound heterozygous cells fell below the range of template concentrations for which linearity had been established, so could not be estimated accurately.

Figure 6.8 Comparison of *cyclin G1* expression in CGR8 mutant and wild-type ES cells using cDNA expression arrays and RT-PCR analysis.



expression array, and the results from the RT-PCR analysis do not show them to be greatly different from one another. In the differentiated cells, the RT-PCR results do not completely agree with those from the expression arrays, as the level of *cyclin G1* RNA did not vary significantly between the wild-type and DDS heterozygous cells according to the expression array (although a small difference was seen), but is seen by RT-PCR analysis to be significantly reduced. This can again be explained by differences in the clones used for the different analyses. The appreciably lower level of *cyclin G1* expression detected in the DDS compound heterozygous cells by expression array is reflected in the results obtained by RT-PCR. Although cyclins are so named due to their varying levels at different points in the cell cycle, this cyclic behaviour is controlled at the protein, rather than RNA, level, so the differences seen here can not be attributed to an indirect effect of the mutations on the proliferative state of these cells.

The gene for heat shock protein 86 (*Hsp86* - also called *Hsp90α*) was seen to be expressed at a higher level in undifferentiated than differentiated wild-type cells using the expression array. This is confirmed in the wild-type by the RT-PCR results (figure 6.9). However, the levels of *Hsp86* RNA seen in undifferentiated mutant cells are far lower, according to RT-PCR. As very little WT1 protein was detected in undifferentiated ES cells (section 4.2.4), it seems likely that these differences in *Hsp86* levels are due to some other factor. If this is the case, then the results from the differentiated cells, from both types of analysis, must also be suspect. Expression of heat shock proteins is notoriously sensitive to slight changes in pH, temperature and other environmental factors (Santoro, 2000), and by chance this could have produced an apparently consistent effect in the mutant cells. This is supported by the fact that

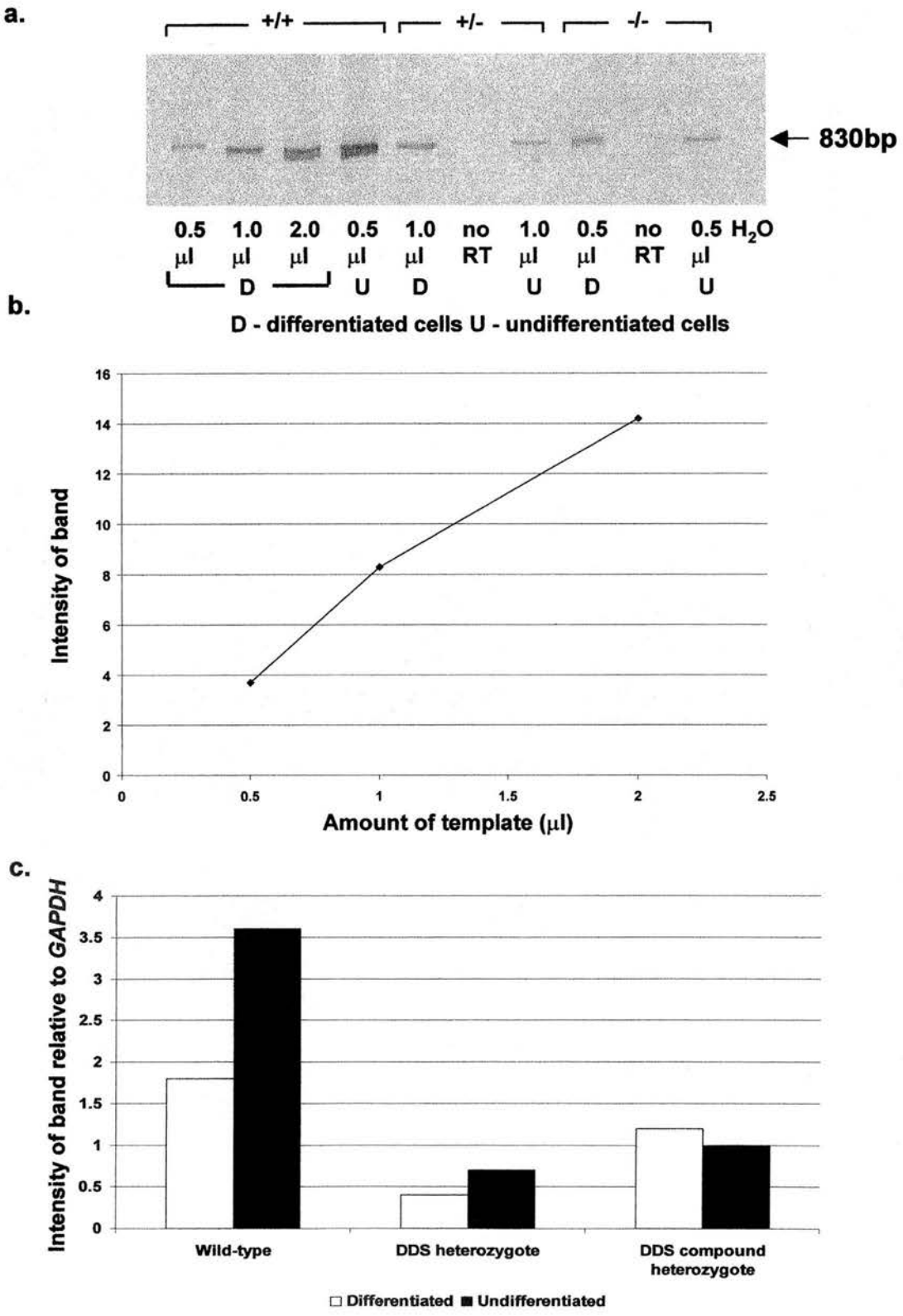
Legend to figure 6.9:

a. Autoradiograph of quantitative RT-PCR, showing intensity of bands produce with *Hsp 86* primers.

b. Standard curve to show that after 16 cycles of PCR, intensity of band produced proportional to amount of template added.

c. Results corrected for amount of template using *GAPDH* PCR results.

Figure 6.9 Comparison of *HSP 86* expression in CGR8 mutant and wild-type ES cells using cDNA expression arrays and RT-PCR analysis.



Hsp86 RNA levels are actually higher in the DDS heterozygous cells than the wild-types according to the expression array, and yet found to be far lower using RT-PCR.This demonstrates exactly why it is important to confirm results obtained by expression arrays using another, independent, method.

Discussion of chapter 6

The comparison of gene expression in retinoic acid-differentiated and undifferentiated ES cells was performed to determine which cell types are being produced by this differentiation process. This was considered interesting, as the vast majority of cells express WT1 after differentiation (section 4.3.4), which is surprising, bearing in mind that WT1 expression is extremely limited during development and adult life (Pritchard-Jones *et al.*, 1990; Buckler *et al.*, 1991; Armstrong *et al.*, 1992). Visual examination of the differentiated cell cultures by light microscope revealed a very morphologically heterogeneous population, so it is unlikely that the uniformity of WT1 expression is due to all the cells differentiating along the same pathway.

Most of the genes seen to be at a higher level in undifferentiated ES cells (see table 6.1) are associated with proliferating cells, or known to be downregulated upon differentiation, whether spontaneous or RA-mediated. For example, there is a strong link between *B-myb* expression and high proliferative ability (Sitzmann *et al.*, 1996) and cyclins such as *B1* would be expected to be expressed at lower levels in more differentiated cells which are not cycling as rapidly (Hanley-Hyde *et al.*, 1992). The

reduction in *B-myb* expression may also be due to transcriptional repression, mediated through E2F (Lee *et al.*, 1998a). Fibroblast growth factor 4 (*FGF-4*) has previously been shown to be downregulated upon differentiation of F9 embryonic carcinoma (EC) cells (Velcich *et al.*, 1989) and RA-induced differentiation of ES cells (Kelly and Rizzino, 2000). Expression of *p53* is known to decrease upon differentiation of ES cells (Sabapathy *et al.*, 1997). Levels of *MSH2* expression have been found to decrease upon retinoic acid-mediated differentiation of human neuroblastoma cell line SH-SY5Y (Belloni *et al.*, 1999) and both *Hsp 90*α and β are downregulated upon differentiation of human EC cells with all-trans retinoic acid (Yamada *et al.*, 2000), as are levels of glutathione S-transferases (Faria *et al.*, 1998). Expression of *c-kit* was found in undifferentiated ES cells by RT-PCR (McClanahan *et al.*, 1993; Schmitt *et al.*, 1991). All-trans retinoic acid has been found to have different effects on haematopoietic cells of different stages (Purton *et al.*, 1999), with an enhancing effect on the differentiation of committed haematopoietic progenitors, but a preventative effect on haematopoietic differentiation in primitive haematopoietic precursors. There is also evidence to suggest that RA suppresses haematopoietic differentiation in ES cells (Menzel, 1999), so, although undifferentiated ES cells express *c-kit*, treatment with RA may bias differentiation away from haematopoietic lineages, and thus away from cells which normally express *c-kit*, leading to the observed lowering of expression in differentiated cells. *IGFBP-2* expression is inversely correlated with degree of differentiation in hepatoblastoma (Akmal *et al.*, 1995), and in the SK-N-BE(2) human neuroblastoma cell line, *IGFBP-2* expression levels decrease after induction of differentiation with retinoic acid (Bernardini *et al.*, 1994).

Some of the genes which appear to be upregulated upon differentiation of ES cells with RA are markers of particular cell lineages. *ZO-1* expression is commonly used as a marker for epithelial lineages (Stevenson *et al.*, 1986), and *Pref-1* for pre-adipocytes (Smas and Sul, 1993). Retinoic acid-mediated differentiation of F9 EC cells has been found to be rapidly followed by an increase in the level of *Hox 2.1* mRNA (Ura and Hirose, 1991) and retinoic acid is known *in vivo* to be necessary for the expression of *Hox 2.4* and establishment of a zone of polarising acitivity (ZPA) during development (Lu *et al.*, 1997). Protein kinase C theta (*PKC-theta*) is predominantly expressed in haematopoietic cell lines (Baier *et al.*, 1994), although if RA does indeed inhibit haematopoietic differentiation, it may be another cell lineage, such as muscle, which is also known to express *PKC-theta* (Mischak *et al.*, 1993) that is responsible for the expression seen here. Support for the presence of muscle lineages comes from the upregulation of *Igf2* in the differentiated cells. Igf-2 is an important modulator of muscle growth and differentiation (Pedone *et al.*, 1994) and has been found to rise to high levels during myogenic differentiation from ES cells (McKarney *et al.*, 1997). The zyxin protein contains a LIM motif, a common feature of many proteins playing roles in the regulation of cellular differentiation (Beckerle, 1997). Glutathione peroxidase levels are seen to increase upon differentiation in several cell lines, including a 4-5 fold increase when cultured myotubes are induced to differentiate into skeletal muscle (Franco *et al.*, 1999). This is probably not a cell type-specific phenomenon, but a general response of cells to differentiation. As has already been mentioned, *IGFBP-4* expression is seen in the human foetus in regions where differentiation is occurring (Delhanty *et al.*, 1993). To examine more precisely the nature of the cells which are being produced by this

differentiation protocol, the expression of specific markers for cell lineage could be examined, perhaps through RT-PCR or a technique such as immunofluorescence. This study of RA differentiation of wild-type ES cells by macroarray analysis has suggested that epithelial cells, preadipocytes and muscle cells are produced upon such differentiation.

Since this work was carried out, a study has been published which describes changes in gene expression in D3 ES cells upon differentiation with RA (Kelly and Rizzino, 2000). This work involved the use of the same type of cDNA expression array as used here. Although many similarities were seen between the genes found to be differentially expressed, some differences were also evident. Table 6.5 indicates the similarities in gene expression patterns between the two studies. Many genes, particularly those encoding cytoskeletal and extracellular matrix proteins, were found to alter expression by Kelly and Rizzino but not by myself. This is most likely due to the fact that my analysis of these membranes was performed purely on a visual basis, rather than using image analysis software.

Table 6.5 Genes found to alter expression upon RA-mediated differentiation of ES cells in two independent studies

Higher in undifferentiated cells	**Higher in differentiated cells**
B-myb proto-oncogene	Tight junction protein *ZO-1*
Heat shock protein 86 (*Hsp90α*)	Zyxin
Lung Kruppel-like factor (*LKLF*)	Plasma glutathione peroxidase
Fibroblast growth factor 4 (*FGF-4*)	*TDAG51*
Laminin receptor 1	Pre-adipocyte factor 1 (*Pref-1*)
	Homeobox protein 2.1 (*Hoxb5*)
	Homeobox protein 2.4 (*Hoxb8*)
	Serine protease inhibitor homologue J6
	Insulin-like growth factor 2 (*Igf2*)

The paper does not state which carrier was used for the retinoic acid, which is usually DMSO, as used here, or ethanol. A difference in carrier could account for differences in the results obtained, as could the continued use of LIF during differentiation in the study by Kelly and Rizzino. The use of a different ES cell line, D3 as opposed to CGR8, most likely explains the majority of the differences, though, as different genes are found to be expressed in the undifferentiated cells. Any genes whose expression changes were detected by Kelly and Rizzino (2000) but not myself are likely to be due to the increased sensitivity of their image analysis method, which utilised image analysis software, as opposed to the method used by myself, which consisted of a comparison of the two images solely by visual assessment.

Both studies found a downregulation in *FGF-4* expression levels during differentiation, which Kelly and Rizzino (2000) link to *Oct-3* (also called *Oct-4*) downregulation (*FGF-4* is a known target of Oct-3/4 during early mouse development – reviewed in Lamb and Rizzino, 1998), whereas no *Oct-3/4* expression was seen by myself in either undifferentiated or differentiated cells. This in itself is an indication that my detection method lacks sensitivity, as the expression of *Oct-3/4* in undifferentiated ES cells and its downregulation upon differentiation is a well-established fact (Nichols *et al.*, 1998). The higher level of sensitivity of Kelly and Rizzino's study, may also be attributable to the fact that the hybridisation signals were generally stronger, suggesting that I may have missed some expressed genes. This may also account for the fact that Kelly and Rizzino found many more genes upregulated than downregulated upon differentiation (61 compared with 18), whereas my numbers were approximately equal (12 and 15). Thus, differences in probe strength, cell line and analysis techniques probably account for most of the discrepancies between the two studies.

Comparison by cDNA array of gene expression between RA-differentiated ES cell progeny with DDS mutations in both alleles of *Wt1* and wild-type cells of the same line revealed nine genes that may be transcriptional targets of WT1 (*cyclin G1*, *PGPx*, *RAD23* UV excision repair protein B, *IGFBP-3*, *IGFBP-4*, *Hsp86*, *Tdag51*, *RAD21*, 14-3-3 protein zeta/delta). Some of these showed increased expression in *Wt1* mutant cells (*PGPx*, *Tdag51*, *RAD21*) others decreased expression compared to wild-type cells (*cyclin G1*, *RAD23* UV excision repair protein B, *IGFBP-3*, *IGFBP-4*, *Hsp86*, 14-3-3 protein zeta/delta), providing further evidence that WT1 may act as both an activator and a repressor of transcription.

Cyclin G1 was seen to be expressed at a very low level in both CGR8 and E14 mutant cells, relative to wild-type cells, suggesting that WT1 protein is required, either directly or indirectly, for its transcription. This cyclin is known to be involved in the transition between the G_2 and M phases of the cell cycle and has been shown to be transcriptionally activated by p53 (Okamoto and Beach, 1994). *Cyclin G1* RNA levels have been shown to increase after p53 induction by DNA damage, which does not occur in *p53* -/- cells (Reimer *et al.*, 1999). It may have a role in the facilitation of p53-dependent apoptosis in response to DNA damage (Okamoto and Prives, 1999). *Cyclin G1* has been seen to be overexpressed in breast and prostate cancer cell lines, in which the cell cycle-dependency of its expression is lost (Reimer *et al.*, 1999). In humans, the *cyclin G1* gene is located at chromosome 5q32-q34. In this region, translocations have been detected in human haematopoietic tumours, including CML and ALL, suggesting a possible connection between *cyclin G1* and leukaemogenesis (Endo *et al.*, 1996). As well as this possible link with *WT1*, *cyclin G1* expression is also seen to be high in ovary and kidney, regions in which *WT1* expression is detected (Horne *et al.*, 1996). It is known that some cells require *WT1* expression to get past the G_2/M cell cycle checkpoint, as *WT1* antisense oligonucleotides cause G_2/M arrest in K562 cells (Yamagami *et al.*, 1996). This could be effected through *cyclin G1*, with *WT1* being necessary for *cyclin G1* expression in these cells.

Glutathione peroxidases interact with selenium to remove reactive oxygen species from the body, thus preventing DNA damage (Reviewed in Yu, 1994). Plasma glutathione peroxidase (*PGPx*) is expressed in tissues that are in contact with body fluids, the kidney for example (Brigelius-Flohe, 1999). Unlike WT1, however,

expression is much higher in the adult kidney than the foetal (Maser *et al.*, 1994). It is localised to the proximal tubules, where it is secreted by the epithelial cells (Maser *et al.*, 1994). Obviously, genes that affect the synthesis of proteins like this one will ultimately affect the amount of DNA damage caused to cells by free radicals. Surprisingly, the level of *PGPx* RNA is increased in the *Wt1* mutant cells, which would have a protective effect against DNA damage, and therefore against possible cancer-causing mutations. This would suggest that part of the normal function of WT1 protein is the transcriptional repression of the *PGPx* gene.

The murine B homologue of the yeast *RAD23* UV excision repair protein gene is expressed at a reduced level in CGR8 and E14 *Wt1* mutant cells, so is a candidate for being transcriptionally activated by WT1. This protein is ubiquitously expressed, with elevated levels in the testis (van der Spek *et al.*, 1996). A reduction in the level of such a DNA repair protein would lead to more DNA damage going uncorrected, increasing the chance of a cancer-causing mutation persisting and undergoing replication. No evidence for a deficiency in WT1 causing an increased frequency of mutations in other genes has ever been published, however. A possible link with Wilms' tumour lies in the fact that the human *RAD23* homologue B gene lies on chromosome 3p25, and translocations of chromosome 3p occur in sporadic Wilms' tumours (Walton *et al.*, 1992).

The genes for two members of the insulin-like growth factor binding protein family (IGFBP-3 and -4) have reduced expression in the *Wt1* mutant cells, so may be genes which are transcriptionally activated by WT1. These proteins have growth suppressive functions, as they are responsible for controlling the levels of free insulin-like growth factors (IGF-I & IGF-II), which have mitogenic activity

(Clemmons *et al.*, 1995). Indeed, lower than normal levels of circulating IGFBP-3 are seen in breast cancer patients (Stoll *et al.*, 1997). Expression of *IGFBP-3* is low in the foetus, then increases after birth (Lindenberg-Kortleve *et al.*, 1997). This is the main carrier protein for circulating IGFs in the adult, and is mostly synthesised by the liver, but also in vascular endothelial cells, such as those of the renal capillary system (Lee *et al.*, 1999). Expression is also seen in human granulosa cells, as is WT1 expression (Barreca *et al.*, 1996). In the human foetus, *IGFBP-4* is highly expressed in the kidney, stomach, intestine and lung (Delhanty *et al.*, 1993). In the adult, expression is mainly seen in differentiating cells, but also the proximal tubules of the mature kidney (Lindenberg-Kortleve *et al.*, 1997). *IGFBP*s *1-6* have been found to be expressed in the mesangial cells of the glomerulus, where they may control IGF-I availability, which has been implicated in glomerular hypertrophy and matrix expansion (Grellier, *et al.*, 1996). Strong *IGFBP-4* expression is seen in the stromal cells of the bone marrow, where it may modulate the haematopoietic response to IGFs (Grellier *et al.*, 1995). IGFBPs also are believed to have IGF-independent effects on growth and apoptosis (Grimberg and Cohen, 2000). As well as being expressed in many similar locations to WT1 and being apparently involved in some of the same processes, a connection between Wilms' tumour and *IGFBP-4* has already been established by the finding that chromosomal region 17q12-q21.1, where this gene is located, is linked to the disease in a Wilms' tumour family (Rahman *et al.*, 1996).

The *RAD21* gene is a homologue of a *Saccharomyces pombe* gene known to be involved in the repair of DNA double strand breaks caused by ionising radiation (Yu *et al.*, 1995). Its expression is ubiquitous, but seen at higher levels in the thymus

and testis, in which it may play roles in V(D)J and meiotic recombination, respectively. Apart from expression in the testis, the only connection with WT1 or Wilms' tumour is that the *RAD21* gene is situated on chromosome 8, gains of which have been detected in familial Wilms' tumours (Altura *et al.*, 1996). *RAD21* expression is seen to be raised in both the CGR8 and E14 mutants, and so may normally be repressed by WT1 protein.

Heat shock protein 86, also referred to as 90 alpha (Hsp90α), is a molecular chaperone, involved with the folding and intracellular transport of proteins such as steroid receptors (Pratt *et al.*, 1992). This folding machinery also plays a role in signal transduction, regulating the folding, assembly and disassembly of signalling molecules between active and inactive states, modulating the activity of kinases, receptors and transcription factors (Rutherford and Zuker, 1994). In the adult kidney, *Hsp90* is expressed in the glomerular podocytes, Bowman's epithelia and epithelial cells from the distal tubules to the collecting ducts (Kamatsuda *et al.*, 1996). Expression of *Hsp90α* has been found to be higher in breast cancer tissues than in normal cells, and may play a role in cellular proliferation (Yano *et al.*, 1996). High *Hsp90α* expression has also been found in other cancers, including those of the endometrium and gastro-intestinal tract, and has been discovered to be the target to which anti-cancer drug geldanamycin binds (Whitesell *et al.*, 1994). Expression of all heat shock protein genes, including *90α*, has been found to be higher in AML cells than those from CML or normal peripheral blood. This may relate to the differentiation state or proliferative potential of the cells (Chant *et al.*, 1995). *Hsp90α* expression was found to be higher in wild-type cells than those with DDS

mutations in both *Wt1* alleles, suggesting that WT1 protein may be involved in the transcriptional activation of this gene.

14-3-3 proteins participate in signal transduction and checkpoint control pathways. Their expression in *Xenopus* oocytes has been found to enhance the activity of Raf-1, leading to DNA synthesis and cell division (Fantl *et al.*, 1994). They are ubiquitously expressed, with their highest concentrations found in the brain, and are mainly located in the cytosol (Martin *et al.*, 1994). 14-3-3 zeta occurs in a posttranslationally modified form in the brain called 14-3-3 delta. This modification enhances the protein's protein kinase C inhibition around two-fold (Aitken *et al.*, 1995). Expression of the *14-3-3 zeta/delta* gene was found to be lower in cells with DDS mutations in both alleles of *Wt1* than in wild-type cells, so WT1 may play a role in the transcriptional activation of this gene.

Cross-linking of the T-cell receptor (TCR), followed by activation of this receptor, leads to apoptosis in T-cell hybridomas. TDAG51 is responsible for *fas* expression, and links the TCR stimulation to the fas-mediated apoptotic pathway (Park *et al.*, 1996). The *TDAG51* gene is known to be expressed in multiple foetal and adult tissues (Frank *et al.*, 1999). This gene has significant homology with a gene called *IPL/TSSC3*, which is expressed exclusively from the maternal allele and located in the imprinted gene cluster on human chromosome 11p15.5 (mouse chromosome 7), associated with Beckwith-Wiedemann syndrome (Lee and Feinberg, 1998). Although these genes are very closely related, it cannot be assumed from the expression array evidence that *Wt1* mutation would also lead to changes in expression of *TSSC3*, although if the *TDAG51* cDNA bound to the expression array membrane was from a region very homologous to *TSSC3*, there could be some cross-

hybridisation occurring, so at least part of the signal might come from *TSSC3*. The manufacturers of the expression array used, Clontech, were unable to rule out the possibility that the cDNA sequence representing *TDAG51* on the membrane could also hybridise to *TSSC3* RNA. An examination of *TSSC3* expression by a method such as RT-PCR in *Wt1* mutant cells could therefore be an interesting experiment. If alterations in the expression of this gene were discovered, a link would be provided between *WT1*-related Wilms' tumours and those found in Beckwith-Wiedemann syndrome, which have no genetic alterations of *WT1*. Expression of *TDAG51* is higher in the cells with mutations in both *Wt1* alleles than in the wild-type cells, suggesting that this gene is repressed by WT1, which is surprising, as a gene such as this, which is involved in the promotion of apoptosis, would have a tumour suppressive function.

Of course, the fact that expression of these particular genes is altered in *Wt1* mutants relative to wild-type cells does not necessarily mean that they are all under the direct transcriptional control of WT1, as they could be further downstream in pathways in which WT1 is involved. Also, any of these genes could be strictly regulated at the protein level, making these observed changes in mRNA levels physiologically irrelevant.

Expression of three of these genes (*cyclin G1*, *IGFBP-4*, and *Hsp86*) was subsequently examined using RT-PCR analysis, and two of them, *cyclin G1* and *IGFBP-4*, were confirmed as being affected by the *Wt1* mutation. Both showed lowered expression in cells lacking wild-type WT1 protein, suggesting that WT1 plays a role in the transcriptional activation of these genes. The third, *Hsp 86*, was shown to vary its expression in a pattern inconsistent with transcriptional regulation

by WT1. Heat shock protein expression is extremely sensitive to environmental effects (Santoro, 2000), and the apparent consistency of changes in expression between wild-type and homozygous/compound heterozygous cells found by microarray analysis can be attributed to chance. This emphasises the importance of checking results obtained from expression arrays using independent experimental samples. Obviously, this finding calls into question whether the remaining seven genes suggested by the expression array are genuine targets of WT1. These cannot be assumed to be correct until confirmed, although none of these types of genes are as susceptible to environmental effects as those for heat shock proteins.

The finding that none of the previously suggested putative WT1 target genes (table 1.1) which were present on the expression array showed significant alteration of expression levels in both mutant cell lines was unexpected (table 6.1). Although some of these genes show significant differences between the mutant and wild-type cells in one direction or the other, none of these differences are consistent between the two cell lines. In fact, *Egr1* expression is significantly altered in both CGR8 and E14 mutant cells, but this is seen as a down-regulation in the CGR8 mutants and an upregulation in the E14 cells. Such differences cannot be accounted for by the *Wt1* mutations present, otherwise they would show consistency between the two cell lines. As only one clone was examined from each cell line, these results can be explained by other differences specific to the individual clone tested, perhaps due to the high G418 selection used to produce the E14 DDS homozygotes. High G418 selection selects for clones in which the introduced sequences have been duplicated, presumably through mitotic recombination (see section 1.1). This is likely to affect other coding sequences in the region, with unknown effects. No evidence has

therefore been obtained here for any of these genes being genuine transcriptional targets for WT1.

Many of these genes, including *IGF2* and *PDGF-A*, were originally suggested to be targets of WT1 as they were found to be overexpressed in Wilms' tumours (Fraizer *et al.*, 1987; Reeve *et al.*, 1985; Scott *et al.*, 1985). Putative WT1 binding sites were identified in their 5' and 3' regulatory sequences, and studies involving co-transfection of *WT1* expression vectors with reporter constructs indicated that WT1 was indeed capable of repressing transcription from their promoters (Drummond *et al.*, 1992; Gashler *et al.*, 1993). However, there is much evidence to suggest that such experiments do not reflect the *in vivo* transcriptional activity of the WT1 protein. The magnitude, and even whether WT1 appeared to be acting as an activator or repressor of transcription has been seen to depend on the cell type used, the promoter used to drive *WT1* expression (Reddy and Licht, 1995) and the isoforms of WT1 expressed (Reviewed in Little *et al.*, 1999). These involved transient transfection, in which the WT1 protein is produced at far higher than physiological levels for a short space of time, rather than the continuous, lower levels that would result from the *WT1* promoter. These experiments were also often performed in cells that do not normally express *WT1*, which calls into question their validity as a test system. Indeed, as all of these experiments involved the expression of only one of the 16 WT1 isoforms (section 1.5.2.1), and WT1 function is believed to be affected by isoform ratio, as seen in Frasier syndrome (Barbaux *et al.*, 1997; Klamt *et al.*, 1998), it is hardly surprising that the results are inconsistent.

Examination of the effects of WT1 on transcription of endogenous genes, rather than promoter constructs, found a repressive effect of WT1 on *EGF-R*

transcription, but no effect on the transcription of *EGR1* or *IGF1-R* (Englert *et al.*, 1995). Two that were found to be affected, *EGF-R* (Englert *et al.*, 1995) and *syndecan-1* (Cook *et al.*, 1996), are unfortunately not represented on the expression array, so cannot be examined here. Again, however, although endogenous target gene expression was used, the WT1 was from an expression vector, therefore not all isoforms were represented and expression was not driven by the *Wt1* promoter. The fact that both the WT1 and the target genes are endogenously expressed may make the present examination of target gene expression in *Wt1* mutant cells a more physiologically valid reflection of the *in vivo* situation than any of those previously reported. The fact that none of the genes widely believed to be regulated by WT1 have their expression altered in the mutant cells does not, however, necessarily mean that these are not true transcriptional targets of WT1. It is suggested that transcriptional activation of a target gene by the WT1 protein involves the binding of WT1 to a specific DNA region, as well as interaction with other proteins, basal transcription factors, for example (Reddy and Licht, 1996). This would necessarily involve both the N- and C-terminal regions of WT1, and would be affected by the truncations present in the mutant cells. Transcriptional repression, on the other hand, may not always involve DNA binding at all, but can occur through protein-protein interaction, either by preventing a specific transcription factor binding to DNA, or by interacting with a bound transcription factor and preventing it binding to other proteins. If this were the case, the mutations present in these cells, affecting only the C-terminal region of WT1, would do nothing to prevent repression, as protein-protein interaction is believed to occur via the N-terminus of WT1. This is supported by evidence that found more transcriptional repressor activity in the N-terminal

region of WT1 than in the C-terminal region including the KTS insert (Tajinda *et al.*, 1994). It would also explain why many more genes are shown to be down-regulated in the mutants than are shown to be upregulated. It could be that the WT1 putative target genes shown here to be unaffected by this mutation are real WT1 targets for which the presence of a functional WT1 C-terminal region is unimportant. Although the compound heterozygous cells express very little WT1 mutant protein, some can be detected (figure 4.13), and may be sufficient to affect transcription of some genes. Obviously many genes on the expression array do appear to be repressed by WT1 and there is no reason why WT1 could not effect transcriptional repression in two different ways, only one of which involves DNA binding by the WT1 zinc fingers. An obvious experiment to try to determine the relative importance of the WT1 N- and C-terminal regions would be to compare expression patterns in wild-type cells and the cells with two DDS mutations used here with WT1 null cells. Any gene whose expression is affected by the true null mutations, but not the DDS mutations, may be influenced by the WT1 N-terminal region and not need a functional C-terminus for transcriptional effects.

A comparison of gene expression between wild-type and DDS heterozygous cells was made for the CGR8 line. This comparison was important, as it had previously been shown that the $Wt1^{tmT396}$ mutation can induce symptoms of DDS in mice (Patek *et al.*, 1999). The results of this analysis were divided into three groups: (1) Genes whose expression altered in heterozygous, compound heterozygous and homozygous cells (table 6.4a) are those whose levels are affected when the cell contains less than the full amount of intact WT1 protein made from both copies of the gene. These genes, which include *IGFBP-3*, *IGFBP-4* and *Tdag51*, are unlikely

to be those involved in Wilms' tumourigenesis, as evidence points to a requirement for the second allele of *WT1* to be lost before a Wilms' tumour is produced. This was demonstrated by the genotype of the Wilms' tumour found in one DDS heterozygous chimaera in which an exon 9 skipping event had occurred in the remaining allele of *Wt1* (Patek *et al.*, 1999). On the other hand, heterozygous DDS-type mutations have been identified in human leukaemias (King-Underwood and Pritchard-Jones, 1998), so any genes whose expression is affected in both heterozygotes and cells with DDS mutations in both alleles of *Wt1* could be relevant to leukaemogenesis. Such genes could also be involved in the other, non-malignant, symptoms of DDS, such as the mesangial sclerosis or genitourinary abnormalities. IGFBPs, as previously mentioned, control levels of IGFs, suppressing their mitogenic activity. As the effect seen in heterozygous cells is so large, it may be that IGFBP downregulation is not a causative step in Wilms' tumour development.

(2) Genes shown to be affected by two *Wt1* mutations only (table 6.4b) could possibly be important for Wilms' tumourigenesis. Expression of the RAD23 UV excision repair protein B is downregulated in the cells with two *Wt1* mutations, but not those with one. This is consistent with it being a gene involved in WT1-related tumourigenesis, and lowered expression of a DNA repair protein will obviously allow possible cancer-causing mutations to go unrepaired.

Expression of the *14-3-3 zeta/delta* gene is very low in both E14 homozygotes and CGR8 compound heterozygotes, yet the change in expression in the heterozygote compared with the wild-type is small. These proteins are involved in signal transduction and checkpoint control pathways (Xing *et al.*, 2000), having an inhibitory effect on protein kinase C (Aitken *et al.*, 1995), so the downregulation in

the homozygote and compound heterozygote could remove this inhibition, allowing cells to progress through checkpoints, and again could lead to the perpetuation of cancer-causing mutations.

(3) Genes which are found to be affected in the heterozygotes only (*IGFBP-2*, Golgi 4-transmembrane spanning transporter, BAX membrane isoform alpha, *LAMR1*, *CDC42*, *CTSB*) could be spurious results due to the use of only one clone for this study (table 6.4c). Alternatively, these could be important genes involved in the aetiology of Denys-Drash syndrome, as they are very good candidates for being genes affected by dominant-negative *WT1* mutations. Such mutant proteins exert a dominant effect on gene expression by interacting with the wild-type protein present in the cell (Reddy *et al.*, 1995b). Obviously, as no wild-type protein is present in the compound heterozygous or homozygous cells, these effects will not occur. If interaction between the DDS mutant and wild-type WT1 proteins leads to the binding of the wild-type protein to novel DNA sequences, these genes may not be normal transcriptional targets of WT1, which is why they would appear only in this group.

The majority of genes in this latter group show increased expression in the mutant relative to the wild-type. This would fit in with the above suggestion that the zinc finger region of WT1 is more important for activation than repression of transcription. The mutant protein could bind to and interact with the wild-type protein in such a way that the zinc finger region of the wild-type protein binds to inappropriate target sequences, leading to expression from genes which would not be usual wild-type WT1 targets. It could be inappropriate expression of such genes at particular developmental stages which leads to the genitourinary abnormalities

observed in DDS, and in the mature kidney that leads to extra-cellular matrix deposition and mesangial sclerosis. This would not be through a dominant-negative mechanism, but suggests that the DDS mutation confers a type of gain of function activity which requires the presence of the wild-type protein within the same cell.

IGFBP-2, which is expressed at higher levels in the DDS heterozygote than the wild-type, is found at high levels in the foetus, where expression is localised to differentiating cells (Lindenbergh-Kortleve *et al.*, 1997). Like the other insulin-like growth factor binding proteins previously mentioned, IGFBP-2 is responsible for regulating the levels of IGFs.

Golgi 4-transmembrane spanning transporter (*MTP*), which is also upregulated in heterozygous cells relative to wild-type cells, is a component of intracellular membranes (Cabrita *et al.*, 1999). It is believed to be involved in the transport of nucleosides and related molecules across such membranes. In yeast it can alter the sub-cellular localisation of certain drugs, such as antimetabolites, antibiotics and steroid hormones, thus changing the sensitivity of the cell to such substances (Hogue *et al.*, 1996).

Bax is a homologue of the apoptosis gene *bcl-2*, and the protein products of these two genes form heterodimers together (Oltvai *et al.*, 1993). Bax suppresses tumourigenesis and stimulates apoptosis *in vivo* (Yin *et al.*, 1997). Interestingly, *Bax* expression is shown to be increased in kidneys following acute renal failure, and in stressed renal tubular epithelial cells in culture (Ortiz *et al.*, 2000). Its expression is also higher in DDS heterozygous than wild-type cells.

Laminin molecules are matrix proteins that anchor the basal lamina to the cell surface. Basal laminae surround cells such as epithelial cells, fat cells and

smooth and striated muscle, and the structure of the lamina will vary according to cell type. This may be due to the different laminin receptors present on the different cell types. The basal lamina in the glomerulus of the kidney is of double thickness, due to being secreted by both epithelial and endothelial cells (McCarthy, 1997). It is continuous with both the blood and urinary space and acts as a filter. Lack of laminin receptor (LAMR1), as is seen in these heterozygous cells, could prevent anchorage of the basal lamina to the cells, and secreted matrix proteins would remain unattached. Unfortunately, the podocyte cells in the glomerulus where WT1 is expressed possess a different laminin receptor, one from the integrin family ($\alpha 3\beta 1$). The laminin receptor whose expression is seen to be affected here (LAMR1) is expressed in tubule epithelial cells, mainly in the distal tubules (Baraldi *et al.*, 1994).

As the results from table 6.4 only rely on the study of expression of one heterozygous clone, a study of other clones, ideally from a different cell line, would enable spurious results, caused by other, unknown, genetic changes unique to this clone to be distinguished, and the genes which are genuinely affected by *Wt1* mutation to be determined.

In conclusion, the use of expression arrays combined with RT-PCR has identified two genes, *cyclin G1* and *IGFBP-4*, which are likely direct or indirect transcriptional targets of WT1. Expression array analysis alone has identified six other genes (*PGPx*, *RAD23* UV excision repair protein B, *IGFBP-3*, *IGFBP-4*, *Tdag51*, *RAD21*, 14-3-3 protein zeta/delta) which are possible WT1 targets but remain to be confirmed using an independent technique.

7. Results of *in vivo* studies

7.1 Production of chimaeric mice using DDS compound heterozygous cells

One of the initial aims of the project was to sequentially target ES cells to generate DDS-type mutations in both alleles of *Wt1* and use these to produce chimaeric mice. Achieving germ-line transmission of these mutations was not the object, as Kreidberg *et al* (1993) had demonstrated that *Wt1* null mice died *in utero*, and it was assumed that this mutation, removing the ability of the WT1 protein to bind to DNA, would have a similarly severe effect. It was intended to use a 'chimaeric rescue' approach, which can help elucidate gene function in two ways. Firstly, a high contribution of mutant cells to a particular tissue in which WT1 is essential could lead to developmental abnormalities of that tissue, enabling the pin-pointing of more specific regions within the genitourinary system and other systems in which *Wt1* is expressed during later development. An alternative possibility is that *Wt1* mutant cells would be so poorly suited for colonisation of these tissues, due to a requirement for WT1 in these areas, that the mutant ES cells would be outcompeted by wild-type cells. In this case, the tissues in question would develop normally, but consistently show failure of colonisation by mutant cells.

Unfortunately, despite repeated attempts at micro-injection of the mutant ES cells, no chimaeric offspring were produced. Despite a lack of any coat-colour chimaerism, all offspring (n =20)were tested for chimaerism using glucose phosphate isomerase (GPI) analysis, and none was found in pancreas, spleen, brain, blood,

kidney, gonads, large or small intestine, or lung. Although it is tempting to suggest that the *Wt1* mutations present in the ES cells had such a severe effect that they were totally out-competed by the wild-type cells present and could not colonise any tissue, this is extremely unlikely, as the expression of *Wt1* during development is confined to very specific areas (see section 1.5.3). It is more likely that the lack of chimaeras was due to technical problems. Thus, no information on the role of *Wt1* in murine development was obtained from this experiment.

7.2 Comparison of teratomas produced from DDS mutant and wild-type ES cells

An experimental teratoma is a type of germ cell-derived benign tumour that can be produced by subcutaneous injection of ES cells into a mouse. As teratomas contain derivatives of all three primitive germ layers, experimental teratoma analysis is a useful tool for determining the developmental potential of ES cells *in vivo*. For example, Reubinoff *et al* (2000) used this method to demonstrate the pluripotency of the ES cells that they had derived from human blastocysts. Human ES cells were grafted into SCID mice and produced teratomas containing tissues derived from all three embryonic germ layers. Teratomas have the advantage over chimaeric rescue experiments in that they assess the innate ability of the ES cells to differentiate, without the presence of wild-type cells, which may act in a paracrine manner to prevent effects of the mutation from being apparent. Disadvantages of the experimental teratoma method include the possibility that differences seen between different genotypes of teratoma could be due to genetic changes at loci other than the

locus of interest, although use of different clones of each genotype should make this unlikely. Also, the tumour can grow from a small subset of the injected cells, perhaps ones which have a growth advantage.

Teratomas have also been used to examine gene function by comparison of mutant and wild-type ES cells, particularly in cases where the effect of the null mutation is embryonic lethal. This is true of embryos lacking either N-cadherin alone, or both N- and P-cadherin (Radice *et al.*, 1997). Production of teratomas from ES cells with these genotypes revealed that some differentiation had occurred, with epithelial differentiation being unaffected in the mutant teratomas (Moore *et al*, 1999b). More striated muscle than controls, but no neural tube-like structures were found in the cadherin null teratomas (Moore *et al*, 1999b). More dramatically, when this method was used to examine the potential of β1 integrin null cells to differentiate into various tissues, null cells either did not grow at all, or only produced small teratomas (Bloch *et al*, 1997). This was attributed to a failure of angiogenesis caused by the lack of β1 integrin.

As can been seen from the above examples, teratoma analysis can be used to identify specific developmental processes in which a gene of interest is involved, and is particularly useful in cases of embryonic lethality due to a null mutation. It was therefore decided to utilise this method to compare wild-type, DDS heterozygous and DDS compound heterozygous/ homozygous ES cells, from both CGR8 and E14 cell lines. Cells were injected subcutaneously into both flanks of a mouse from an isogenic strain (129/Ola), with a different genotype of cells being injected into each flank, enabling direct comparisons between tumours to be made. Teratomas were allowed to develop for up to 70 days before being dissected out and analysed, with

the tumours from the same mouse being removed at the same time. Weights of teratomas were compared, then histological analysis was performed on sections stained with haematoxylin and eosin.

No difference was seen between the average weights of teratomas from the different ES cell genotypes (see table 7.1), suggesting that no failure of angiogenesis occurred in any genotype.

Table 7.1: Weights of teratomas produced by ES cells of different genotypes

ES cell genotype	Average teratoma weight (g)	Average tumour growth time (days)
Wild-type (n =10)	1.7 (s.e.m. = 0.35)	34.3 (s.e.m. = 1.60
Heterozygote (n =9)	1.8 (s.e.m. = 0.49)	37.2 (s.e.m. = 4.3)
Homozygote/ Compound heterozygote (n =16)	1.8 (s.e.m. = 0.39)	36.9 (s.e.m. = 3.3)

Histology revealed that derivatives of all three germ layers were present in teratomas produced from ES cells of all three genotypes, with no difference consistent with genotype found between teratomas of different genotype (table 7.2, figure 7.1). From the endoderm, glandular epithelium was found in most of the teratomas; squamous epithelium and neural tissue, from the ectoderm, were also seen in many teratomas. Some mesodermally-derived components, such as muscle, fat, cartilage and bone were also present. Of particular interest is the observation that no difference was found between the three genotypes with regard to formation of muscle. It has been suggested that loss of functional WT1 promotes myogenesis

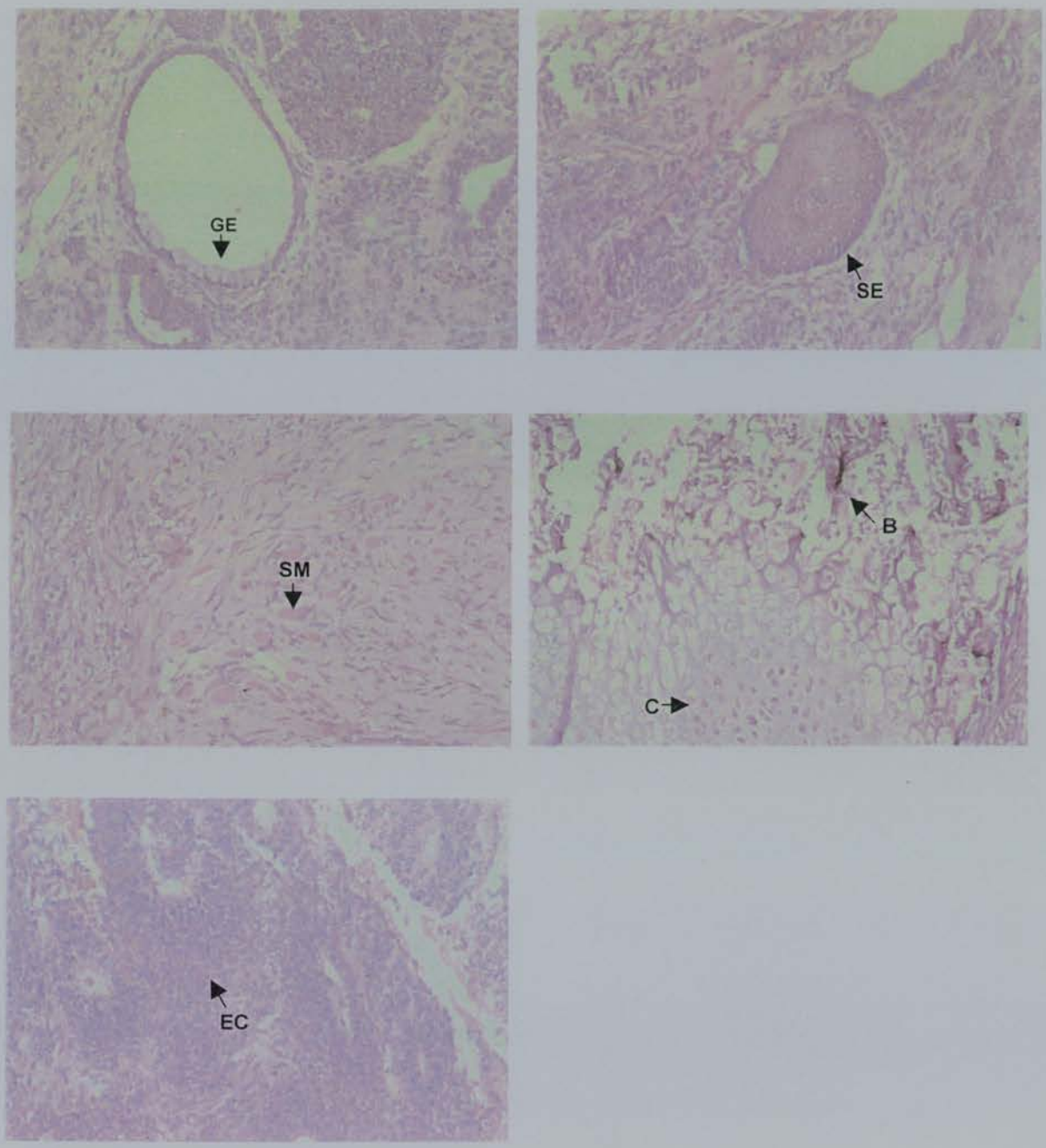


Figure 7.1 Sections of teratomas

All are from teratomas made using CGR8 DDS compound heterozygous cells, which were representative of teratomas produced from cells of all genotypes.
x200
GE – glandular epithelium
SE – squamous epithelium
SM – skeletal muscle
C – cartilage
B – bone
EC – embryonal carcinoma

(Miyagawa *et al*, 1998), but there was no greater prevalence of myogenic differentiation in the teratomas produced from *Wt1* mutant cells.

The results from the teratoma experiment and analysis of embryoid bodies produced from ES cells of the different DDS genotypes (section 5.1), reveal that *Wt1* mutations of this nature do not prevent early differentiation of the three primitive germ layers. This is consistent with the findings of Kreidberg *et al* (1993) when studying *Wt1* null mice. These developed normally except for very specific abnormalities, of the genitourinary system, thoracic cavity, diaphragm, heart and lungs. It is perhaps as expected that mutations in a gene which is not expressed until mouse E9 do not affect embryoid body and teratoma differentiation, which probably reflect the very earliest stages of mouse development. The results of this experiment reveal very little about the role of WT1 in developmental processes, except that it is not required for the differentiation of endoderm, ectoderm and mesoderm *per se*.

Table 7.2 Composition of teratomas

Cell line	Genotype	Age (dys)	Weight (g)	EC	Epith.	Muscle	Fat	Neuro	Cartilage	Bone
CGR8	wt	31	2.1	Y	G	N	N	Y	Y	N
CGR8	wt	31	1.7	Y	N	N	Y	Y	Y	Y
CGR8	wt	42	3.3	Y	G,S	N	N	N	N	N
CGR8	wt	36	2.7	Y	G	N	N	Y	Y	N
CGR8	wt	36	1.6	Y	G	N	N	Y	Y	N
CGR8	wt	32	2.8	Y	G,S	Y	N	Y	Y	N
CGR8	wt	32	1.3	Y	G	N	N	N	N	N
CGR8	wt	43	0.2	Y	G	N	N	N	N	N
CGR8	het	31	3.8	Y	G,S	N	N	N	Y	N
CGR8	het	31	1.2	Y	G,S	N	N	N	N	N
CGR8	het	36	0.1	N	G	N	N	Y	N	N
CGR8	het	32	1.6	Y	G,S	N	N	Y	N	N
CGR8	het	70	0.1	N	G	N	N	Y	N	N
CGR8	het	43	0.2	Y	N	N	N	N	N	N
CGR8	ch	42	0.4	Y	G,S	N	N	N	N	N
CGR8	ch	36	0.1	Y	G	N	N	Y	Y	N
CGR8	ch	66	3.3	Y	G	N	N	N	N	N
CGR8	ch	66	1.9	Y	G,S	N	N	Y	N	N
CGR8	ch	70	0.1	N	G	Y	N	Y	N	N
E14	wt	28	0.5	Y	G,S	N	N	Y	N	N
E14	wt	32	0.2	Y	G	Y	N	Y	Y	N
E14	het	28	2.2	Y	G	N	N	N	Y	N
E14	het	32	3.4	Y	G	Y	N	Y	Y	N
E14	het	32	3.1	Y	G,S	Y	N	Y	N	N
E14	hom	28	1.1	Y	G,S	N	N	N	Y	N
E14	hom	28	2.2	Y	G,S	Y	N	N	Y	N
E14	hom	28	0.6	Y	G	N	N	N	Y	N
E14	hom	28	0.5	Y	G,S	N	N	Y	Y	N
E14	hom	28	1.1	Y	G,S	N	N	Y	Y	N
E14	hom	28	1.7	Y	G,S	N	N	Y	N	N
E14	hom	26	2.9	Y	G,S	N	N	Y	Y	Y
E14	hom	26	4.8	Y	G,S	N	N	Y	N	N
E14	hom	26	2.6	Y	G,S	N	N	Y	N	N
E14	hom	32	4.7	Y	G,S	Y	N	Y	Y	N
E14	hom	32	0.4	Y	G	Y	Y	Y	N	N

Age – time for which tumour grown

Y – tissue present

N – tissue absent

Epith. – epithelium: G- glandular, S – squamous

EC – embryonal carcinoma

Neuro – neural tissue

Genotypes:

wt – wild-type

het – DDS heterozygous

ch – DDS compound heterozygous

hom – DDS homozygous

8. Conclusions and future directions

1. *Wt1* is only expressed at very low levels in undifferentiated ES cells. Differentiation with all-trans retinoic acid causes *Wt1* expression levels to increase. Evidence is presented here that suggests that this expression is due to the differentiation status of the cells, rather than a direct effect of the RA on *Wt1* expression. The induction of *Wt1* expression by retinoic acid provides a useful tool for studying *Wt1* using techniques which require high levels of RNA or protein. To fully understand the mechanism through which *Wt1* is expressed, however, the cell types produced during this differentiation process need to be determined. This could be carried out *in situ*, using antibodies against cell surface markers known to be specific to particular cell lineages.

2. WT1 protein is found at lower levels in DDS compound heterozygous differentiated ES cells than in wild-type or DDS heterozygous cells of the same line, differentiated in the same manner. To determine whether the situation is the same as had previously been found in DDS homozygous cells, in which the levels of *Wt1* mRNA were higher than would be expected from the level of WT1 protein detected, Northern analysis must successfully be performed on these cells.

3. From the comparison of WT1 protein subnuclear localisation in differentiated wild-type and DDS heterozygous cells, it can be concluded that this mutation

does not lead to the sequestration of wild-type WT1 protein into the splicing domains.

4. The ratios of the *Wt1* isoforms possessing and lacking the first alternatively spliced region cover the same range in differentiated ES cells as in other murine tissues. Ratios of the different KTS isoforms, on the other hand, are different in ES cells than in tissues previously examined, with an excess of isoforms lacking the KTS insert found in ES cell progeny. An examination of these isoforms in foetal mouse tissues would be interesting to determine whether this phenomenon is unique to ES cell progeny, or a property of foetal mouse tissue in general.

5. The presence of a DDS-type truncation mutation in one or both alleles of *Wt1* does not significantly affect the ability of ES cells to form embryoid bodies *in vitro*, or the range of cell types produced upon their differentiation. This could be examined in closer detail using lineage-specific markers.

6. The DDS-type truncation mutation studied here exerts an effect on haematopoietic differentiation *in vitro* when present in either one or both *Wt1* alleles in an ES cell. Closer examination of the haematopoietic lineages present in the CFU-A colonies produced by mutant and wild-type cells would indicate whether the observed delay in differentiation is accompanied by either a lack or an excess of a particular lineage at later stages. An *in vivo* study of the ability of dissociated mutant and wild-type embryoid bodies different time-points to

achieve long-term repopulation of lethally irradiated mice could provide further evidence for the existence of a delay caused by *Wt1* mutation.

7. Expression of the genes for cyclin G1 and IGFBP4 is affected by the presence of DDS-type mutations in both alleles of *Wt1*. These genes could be direct transcriptional targets of the WT1 protein, or lie further downstream in a pathway involving WT1. The relevance of these genes as transcriptional targets of WT1 can be examined by looking for potential WT1 binding sites in their control regions, and by the use of reporter constructs to assess the ability of WT1 to repress transcription from their promoters. This last method, however, would require careful thought, to ensure that conditions are as physiological as possible. A comparison of expression levels in these two genes in Wilms' tumours with and without *Wt1* mutations could help to determine the importance of these genes in Wilms' tumourigenesis.

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Appendix I - solutions

A. Solutions for embryonic stem cell culture

Medium CM
BHK-21 Glasgow Modified Eagle's Medium (Life Technologies) supplemented with:
Non-essential amino acids:
0.1mM glycine
0.1mM L-alanine
0.1mM L-aspartic acid
0.1mM L-asparagine
0.1mM L-glutamic acid
0.2mM L-proline
0.2mM L-serine
1.0mM sodium pyruvate
5% (v/v) foetal calf serum
5% (v/v) newborn calf serum

Medium CMβ
Medium CM, with the addition of:
0.1mM β-mercaptoethanol

Medium EFNβ
Medium CMβ, without the addition of sodium pyruvate and non-essential amino acids.

Standard tissue culture medium
Medium CMβ, with the addition of sufficient LIF to prevent ES cell differentiation. Currently using stock at 0.2% v/v.

TVP
0.025% (w/v) trypsin
1mM Na_2-EDTA
1% chick serum (Life Technologies)
in PBS

Fixative for metaphase spreads
2:1 methanol: acetic acid (v:v)

2X stock for CFU-A assay
21ml MEM stock
25ml horse serum
1ml glutamine
3ml sodium bicarbonate

Bouin's fluid
4% formaldehyde
2% acetic acid
1% picric acid

B. Solutions for vector and probe preparation

S.O.C. medium

1M KCl	0.25ml
1M NaCl	1.0ml
bactotryptone	2.0g
yeast extract	0.5g

Stir to dissolve, autoclave, then cool to room temperature. Then add:

2M Mg^{++} stock (1M $MgCl_2 \bullet 6H_2O$ + 1M $MgSO_4 \bullet 7H_2O$, filter sterilised)	1ml
2M glucose stock	1ml

Filter through 0.2μm filter.

L-Amp broth (litre)
10g tryptone
10g NaCl
5g yeast extract
50mg ampicillin

L- Amp agar
1 litre L-Amp broth
12g Bacto-Agar (Difco)

TE buffer
10mM Tris-HCl (pH 7.4)
1mM Na_2-EDTA (pH 8.0)

TBE buffer x1
0.09M Tris
0.09M Boric acid
0.002M Na_2-EDTA

Electrophoresis loading buffer
1x TBE
10% glycerol
0.25% mg bromophenol blue

C. Solutions for DNA analysis

DNA lysis buffer
50mM Tris pH 7.5
50mM Na_2-EDTA
100mM NaCl
5mM DTT
0.4mM Spermidine phosphate
1% SDS

DNA loading buffer
1x TBE
10% glycerol
0.25% mg bromophenol blue

Denaturing solution
1.5M NaCl
0.5M NaOH
pH 7.0

20X SSC
3M NaCl
0.3M Sodium citrate

Hybridisation solution
5g dextran sulphate (in 50ml)
6X SSC
1% SDS

Washing solution
1% SDS
0.2X SSC

6% denaturing gel
40% 19:1 acylamide/bisacrylamide 75ml
10X TBE 50ml
8M urea

Denaturing gel loading buffer
400µl 0.5M Na_2-EDTA
9.5ml formamide
5mg xylene cyanol
5mg bromophenol blue

D. Solutions for RNA analysis

DEPC-treated water
0.01% (v/v) diethylpyrocarbonate
Left to stand overnight, then autoclaved.

RNA loading dye
1x TBE
10% glycerol
0.25% mg bromophenol blue
(made up in DEPC-treated water)

RNA sample buffer
50% formamide
1.7x MOPS
40% formaldehyde

MOPS buffer
0.2M MOPS
0.05M NaOAc
0.01M Na_2-EDTA
pH 7.0

RNA agarose gel
1% agarose
1x MOPS buffer
6.5% formaldehyde

Transfer buffer
0.04M NaOH
0.5M NaCl

10X DNaseI buffer
400mM Tris-HCl (pH 7.5)
100mM NACl
60mM $MgCl_2$

10X DNaseI temination solution
0.1M EDTA (pH 8.0)
1mg/ml glycogen

E. Solutions for protein analysis

Sucrose buffer (x10 stock made up without sucrose)
600mM KCl
150mM NaCl
5mM Spermidine
1.5mM Spermine
20mM Na_2-EDTA
Dilute 1/10 for x1 stock, then add 300mM sucrose, and 1 complete protease inhibitor cocktail tablet.

Resolving gel (20ml)	10% gel	8% gel
ddw	11.9ml	13.9ml
29:1 30% acrylamide/bisacrylamide	10.0ml	8.0ml
1.5M Tris pH 8.8	7.5ml	7.5ml
10% SDS	0.3ml	0.3ml
10% APS	0.3ml	0.3ml
TEMED	21µl	18µl

Stacking gel (20ml 5% gel)	
ddw	13.6ml
29:1 30% acrylamide/bisacrylamide	3.4ml
1M Tris (pH 6.8)	0.2ml
10% SDS	0.2ml
10% APS	0.2ml
TEMED	20µl

TGS running buffer
25mM Tris
200mM glycine
3mM SDS

x2 loading buffer
4% SDS
20% glycerol
120 mM Tris (pH 6.8)
200 mM DTT
0.02% bromophenol blue

Transfer buffer (x10)
0.25M Tris
2M glycine
3mM SDS
pH 8.6

TBST
20mM Tris
137mM NaCl
pH 7.6
0.1% Tween 20

Immunofluorescence blocking solution
6.7% glycerol
2% BSA
0.05% Tween 20
0.02% Sodium azide

F. Solutions for *in vivo* methods

GPI sample buffer
1.9g Triethanolamine hydrochloride
(in 200ml) pH 7.6
60mg DTT (Sigma)
100mg BSA fraction V (Sigma)
400mg Digitonin (sigma)

GPI gel fixative
15% glycerol
3% glacial acetic acid

Appendix II – PCR primer sequences and conditions

1. Primers for sequencing WTV2

5' 3'

Forward (WTV2F/DS26393) TGA AAC CAT TCC AGT GTA AAA C

Reverse (WTV2R) CGG TGG ATG TGG AAT GTG T

94° 3min

94° 1min

58° 1min x30

72° 1min

72° 8min

2. GAPDH primers for standardisation of RT-PCRs

Product size: 570bp

Forward (GAPDH1) AGT CAG CCG CAT CTT CTT

Reverse (GAPDH4) CCA AAG TTG TCA TGG ATG ACC T

94° 5min

94° 30sec

58° 30sec x16

72° 30sec

72° 5min

3. WT1 primers for examination of isoform ratios

a. +/- exon 5 (Moore *et al*, 1999)

Product sizes: 192, 243bp

Forward (K447) GAC GCC CTA CAG CAG TGA CAA

Reverse (K448) CGC ACA TCC TGA ACT CAT ACA G

94° 5min

94° 30sec

55° 40sec x35

72° 1min

72° 5min

b. +/- KTS (Moore *et al*, 1999)

Product sizes: 106, 115bp

Forward (J421) CCA CAC CAG GAC TCA TAC AG

Reverse (J420) TGC ATG TTG TGA TGG CGG AC

94° 2min 30sec

94° 30sec

55° 45sec x35

72° 1min

72° 9min

Requires 2mM $MgCl_2$

c. +/- KTS primers to confirm genotype of targeted cells

Product sizes 173, 164bp

Forward (DDS1) GTG TAA AAC TTG TCA GCG AAA G
Reverse (DDS2) TAC GTA CAA CAC TAC CGC CTG

94° 5min
94° 30sec
55° 45sec x35
72° 1min
72° 5min

4. Primers for haematopoiesis RT-PCRs

a. *c-kit* (Schmitt *et al*, 1991)

Product size: 1069bp

Forward (SCFRS) CAA CAG CAA TGG CCT CAC GAG T
Reverse (SCFRA) GTG GTA CAC CTT TGC TCT GCT C

94° 5min
94° 1min
65° 1min x14
72° 2min
72° 5min

b. G-CSF receptor (Schmitt *et al*, 1991)

Product size: 567bp

Forward (GCSFRS) CCC CTC AAA CCT ATC CTG CCT C
Reverse (GCSFRA) TCC AGG CAG AGA TGA GCG AAT G

94° 5min

94° 1min

65° 1min x16

72° 2min

72° 5min

c. Foetal β-globin (Schmitt *et al*, 1991)

Product size: 415bp

Forward (GLOBFS) AAC CCT CAT CAA TGG CCT GTG G

Reverse (GLOBFA) TCA GTG GTA CTT GTG GGA CAG C

94° 5min

94° 1min

65° 1min x16

72° 1min

72° 2min

d. Adult β-globin (Schmitt *et al*, 1991)

Product size: 444bp

Forward (GLOBAS) ATG GTG CAC CTG ACT GAT GCT G

Reverse (GLOBAA) GGT TTA GTG GTA CTT GTG AGC C

94° 5min

94° 1min

65° 1min x16

72° 2min

72° 5min

5. Primers for RT-PCRs to confirm mouse Atlas expression results

(numbers of cycles represent where PCR was found to be in log phase for amount of template used)

a. Insulin-like growth factor binding protein 4

Product size: 735bp

Forward (IGFBP4S) TGG GTT GCG AGG AGT TGG T
Reverse (IGFBPSA) CCA GGA CTC AGA CTC CAA

94° 5min
94° 1min
58° 1min x16
72° 1min
72° 5min

b. Heat shock protein 86

Product size: 830bp

Forward (HSP86S) CCA GTT AAT GTC CTT GAT CA
Reverse (HSP86A) CAT TAG TGA TGT CAT CAG GA

94° 5min
94° 1min
55° 1min x16
72° 1min
72° 5min

c. Cyclin G1

Product size: 803bp

Forward (CYCG1S) ACT GAC AAC TGA CTC TCA GA

Reverse (CYCG1A) GTG CTT CAG TTG CCG TGC AGT

94° 5min

94° 1min

58° 1min x12

72° 1min

72° 5min

Appendix III – suppliers

Amersham Pharmacia
Amersham Place,
Little Chalfont, Buckinghamshire
HP7 9NA

Anachem Ltd.
20 Charles Street,
Luton, Bedfordshire
LU2 0EB

Autogen Bioclear UK Ltd.
Holly Ditch Farm,
Mile Elm,
Calne, Wiltshire
SN11 0PY

BioRad Laboratories Ltd.
BioRad House
Maylands Avenue,
Hemel Hempstead, Hertfordshire
HP2 7TD

Clontech UK Ltd.
Unit 2, Intec 2
Wade Road,
Basingstoke, Hampshire
RG24 8NE

Corning Costar UK Ltd.
1 The Valley Centre,
Gordon Road,
High Wycombe, Buckinghamshire
HP13 6EQ

Dako Ltd.
Denmark House
Angel Drove,
Ely, Cambridge
CB7 4ET

Difco Laboratories Ltd.
P.O. Box 14B
Central Avenue,
East Molesley, Surrey
KT8 2SE

Fisher Scientific UK
Bishop Meadow Road
Loughborough, Leicestershire
LE11 5RG

Flowgen
Lynn Lane,
Shenstone,
Lichfield, Staffordshire
WS14 0EE

Genosys (Sigma-Genosys Ltd.)
London Road,
Pampisford, Cambridgeshire
CB2 4EF

Greiner Labortechnik Ltd.
Brunel Way,
Stonehouse, Gloucestershire
GL10 3SX

Helena Biosciences
Colima Avenue,
Sunderland Enterprise Park,
Sunderland, Tyne and Wear
SR5 3XB

Hybaid Ltd.
Action Court
Ashford Road,
Ashford, Middlesex
TW15 1XB

ICN Pharmaceuticals Ltd.
1 Elmwood
Chineham Business Park,
Basingstoke, Hampshire
RG24 8WG

Life Technologies Ltd.
3 Fountain Drive,
Inchinnan Business Park,
Paisley
PA4 9RF

Mackay and Lynn Ltd.
2 West Bryson Road,
Edinburgh,
EH11 1EH

Merck Ltd.
Merck House,
Poole, Dorset
BH15 1TD

Promega UK Ltd.
Delta House,
Chilworth Research Centre,
Southampton
SO16 7NS

Roche Diagnostics Ltd.
Bell Lane,
Lewes, East Sussex
BN7 1LG

Scottish Antibody Production Unit
(SAPU)
Law Hospital,
Carluke, Lanarkshire
ML8 5ES

Sigma-Aldrich Company Ltd.
Poole, Dorset
BH12 4QH

Stratagene Europe
Gebouw California,
Hogehilweg 15,
1101 CB Amsterdam Zuidoost,
The Netherlands

Vector Laboratories Ltd.
3 Accent Park,
Bakewell Road,
Orton Southgate, Peterborough
PE2 6XS

Wolf Laboratories Ltd.
Unit J, Lancaster Road,
Pocklington Industrial Estate,
York
YO42 2NR